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THE ADRENAL CORTEX AND HOMEOSTASIS[1]

GEORGE SAYERS

From the Department of Pharmacology, University of Utah

SALT LAKE CITY, UTAH

THE ADRENAL CORTEX

INDICES OF RATE OF SECRETION OF ADRENOCORTICAL HORMONE 244
I. Alterations in the Adrenal Cortex 244
1) Gross anatomical 244
2) Histological 244
(a) Lipids 245
(b) Localization of secretory zones 245
(c) 'Ketosteroids' 245
3) Chemical 247
(a) Cholesterol 247
(b) Ascorbic acid 248
(c) Phosphorus 250
II. Alterations in the Organism 250
1) Lymphocytes 250
2) Eosinophils 252
3) Metabolic 253
III. Urinary Steroids 253
1) Urinary biocorticoids 254
2) Urinary chemocorticoids 254
(a) Phosphomolybdic acid 255
(b) Copper 255
(c) Formaldehyde 255
3) Urinary 17-ketosteroids 258
IV. Blood Corticoids 259
1) Blood biocorticoids 259
2) Blood chemocorticoids 260
ESTIMATES OF RATE OF SECRETION OF THE ADRENOCORTICAL HORMONE 260
ESTIMATES OF RATE OF 'UTILIZATION' AND DEGRADATION OF THE ADRENOCORTICAL HORMONE 262
NATURE OF SECRETION OF THE ADRENAL CORTEX 262
I. Multisteroid Concept 263
II. 'N' and 'S' Hormone Concept 263
III. Autonomy Concept 264
IV. Unitarian Concept 265

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REGULATION OF RATE OF SECRETION OF THE ADRENOCORTICAL HORMONE 266
I. Pituitary Regulation of Secretory Activity of THE Adrenal Cortex 266
II. Regulation of Pituitary Adrenocorticotrophic Activity 268
1) Peripheral-humoral mechanism 268
2) Central-neural and central neuro-humoral mechanism 273
3) Role of the sympatho-adrenal system 274
CLASSIFICATION OF RESPONSES OF THE ADRENAL CORTEX TO STRESS 276
Type I. A Sudden Temporary Period of Stress 277
Type II. A Very Gradual Change in the Internal or External Environment 280
Type III. An Intense Continuous Stress Ending in Death 280
Type IV. Recovery From a Period of Severe Stress 280
Type V. Adaptation to Stress 281
Types VI, VII, VIII and IX 281
THE ORGANISM
INCREASED RATE OF UTILIZATION OF CORTICAL HORMONE DURING STRESS 282
METABOLIC CHANGES IN STRESS: ROLE OF THE ADRENAL CORTEX 284
I. Carbohydrate Metabolism 285
II. Protein Metabolism 287
III. Fat Metabolism 288
IV. Electrolyte Metabolism 288
RESISTANCE TO STRESS 289
I. Decreased Resistance to Stress after Adrenalectomy 290
II. Does Stress Induce Hypocorticism? 290
1) Does a normal pituitary-adrenal system ever fail to meet the demands of the tissues for cortical hormone in severe stress? 290
2) 'Exhaustion' of the adrenal cortex 291
3) 'Exhaustion' of the adenohypophysis 292
III. Mode of Action of Cortical Hormone in Increasing Resistance to Stress 294
1) Detoxification 294
2) Cardiovascular dynamics 294
3) Mobilization of antibodies 294
4) Ubiquity concept 296
ADAPTATION TO STRESS 296
ARE PATHOLOGICAL CHANGES INDUCED BY THE HYPERACTIVITY OF THE ADRENAL CORTEX WHICH ACCOMPANIES STRESS? 298
I. The 'Adaptation Syndrome' 298
II. 'Excessive' Production of Adrenocortical Hormone 299
III. DCA Toxicity 300
IV. Toxicity of ACE and 11,17-Oxysteroids 302
V. Toxicity of Anterior Pituitary Extracts and Purified ACTH 302
VI. Pathology of Cushing's Syndrome 303
VII. Production of 'Diseases of Adaptation' in Experimental Animals 303
VIII. Adrenal Cortex and Essential Hypertension 304
IX. Allergic Basis of Rheumatic Heart Lesions and Periarteritis Nodosa 306
X. Deficiency of 11,17-Oxysteroids Induced by DCA 307
XI. Therapy of Collagen Diseases with Cortisone and ACTH 307

THE ROLE OF NUMEROUS BUFFER ORGANS, in particular the sympatho-adrenal system, in endowing the higher organism with relative freedom from a constantly changing environment has been lucidly presented by Cannon in *The Wisdom of the Body* (47). In the decade that has elapsed since the appearance of the second edition of this publication the adrenal cortex has been demonstrated to occupy a key position in the complex physiological processes concerned with the maintenance of the steady state. Indeed, the pituitary adrenocortical system is now gen-

erally recognized to play an even more ubiquitous role in homeostasis than the sympatho-adrenal system. A most striking feature of the adrenal cortex, as an organ of homeostasis, is its ability to endow the organism with resistance not to a few, but to all types of stress. This ubiquitous role of the gland must be taken into account by any theory which attempts to explain its mode of action in increasing the resistance of the organism to stress.

In this review the subject of the adrenal cortex and homeostasis has been divided into two phases: *1*) the effect of stress upon the secretory activity of the adrenal cortex, and *2*) the influence of the secretion of the gland on bodily functions.

Regarding the first phase of the subject, it is possible, as a result of the advances of the past 20 years, to present a satisfactory although admittedly incomplete account of the secretory activities of the pituitary and the adrenal cortex.

In contrast, there is a glaring deficiency in our knowledge concerning the second phase, the mechanism of action of the cortical hormone on the target cells, an action intimately concerned with the resistance of the organism to stress. Unfortunately, progress has been hindered by the tendency of specialists to place undue emphasis upon specific functions of the cortical hormone. In addition, scant attention has been given to certain fundamental principles of adrenocortical physiology,—principles emphasized recently by Ingle (199). Two of these require mention at this juncture.

First, no fixed amount of cortical hormone represents a physiological dose; the needs of the tissues vary widely, depending upon the activity and the environment of the organism. A dose which induces hypercorticism under optimal conditions, i.e., a dose which might be characterized as pharmacological, may barely satisfy the needs of an organism vigorously engaged in homeostatic activities. In other words, a dose which is pharmacological under optimal conditions may be less than adequate for the needs of the tissues during stress and may not prevent the development of hypocorticism. Thus the metabolic responses to the same dose of hormone under the varying circumstances of hypercorticism, eucorticism and hypocorticism may be entirely different.

Second, the actions of the cortical hormone are determined in no small measure by the functional status of the tissue cells themselves. How else can one explain the actions of a hormone which can promote water retention or diuresis, sodium conservation or excretion, anabolism or catabolism, and inhibition or acceleration of glucose utilization? Further progress in the study of the adrenal cortex in homeostasis demands wider application of such fundamental concepts concerning dose and response.

It is of some importance to realize that the cortical hormone plays a general supportive rather than an initiating role in bodily processes. Whereas the adrenal medulla initiates cellular and metabolic changes in response to an emergency, the adrenal cortex plays a passive role and makes it possible for the various regulatory systems to expend the additional effort necessary for homeostatic adjustment. The adrenalectomized animal fails under any circumstance which calls for resistance to change, regardless of the buffer system or systems involved. This point of view emphasizes the capacity of the hormone to 'normalize' the *milieu intérieur* regardless of the direction of the disturbance. The role of the adrenal cortex is indeed a ubiquitous one.

However, it is unlikely that the very numerous and diversified physiological

functions of the cortical hormone are paralleled by as many different actions on intracellular biochemical processes. The law of parsimony necessitates the hope that these functions are the varied expressions of a ubiquitous single action, albeit complex, concerned with the mobilization of energy for cellular work, regardless of the type of cell involved or the nature of the work performed. On this point, however, there is practically no cogent evidence.

THE ADRENAL CORTEX

Indices of Rate of Secretion of Adrenocortical Hormone

There is no entirely satisfactory method for measuring the secretory activity of the adrenal cortex. With the exception of the method of Vogt (487), which unfortunately is very limited in its applications, the methods are indirect in their approach. The reviewer is unaware of any critical evaluation of the large number of technics now employed in regard to specificity, sensitivity, accuracy and applicability.

Indices for the assessment of adrenocortical activity may be classified under four general headings, according to whether measurement is made of *1*) alterations in the adrenal cortex, *2*) alterations in the organism, *3*) rate of excretion of steroids in the urine, or *4*) titer of corticoids[3] in the blood.

I. *Alterations in the Adrenal Cortex.* 1) GROSS ANATOMICAL. Continued hyperactivity of the adrenal cortex is associated with an increase in the size of the gland. Tepperman *et al.* (459), in an extensive review of the literature, cite a large number of stressful conditions which are accompanied by an enlargement of the adrenal cortex. Enlargement of the gland occurs within 6 to 24 hours after initiation of a severe stress (115, 190). Apparently new protoplasm is laid down at an early stage following stimulation because the increase in the weight of the adrenal, which follows 12 hours of muscular activity, cannot be entirely accounted for by accumulation of water (190).

Wet or dry weight of the adrenal is a good index of adrenocortical activity in experiments of more than a few days' duration. The simplicity of the technic is of considerable advantage. In the reviewer's experience there has been good correlation between adrenal weight and the expected activity of the adrenal cortex, as judged by the severity and duration of the stress to which an animal has been exposed. Furthermore, adrenal weight increases proportionately to the dose of administered adrenocorticotrophic hormone (ACTH) (256, 403).

In conditions of severe and protracted stress, the gland appears not only grossly enlarged but reddish brown in color instead of yellow, a change due to loss of cytoplasmic lipid (399). Hemorrhagic areas may sometimes be seen on the surface of adrenals which have been extremely active.

2) HISTOLOGICAL. Histochemical technics have been employed to *1*) measure fluctuations in the secretory activity of the adrenal cortex, and to *2*) differentiate the zones of the adrenal cortex in regard to functional activity.

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[3] Corticoid, as the term is used in this review, refers to a substance which has not been unequivocally identified as a cortical hormone, but which has certain chemical or biological properties characteristic of the crystalline cortical steroids.

a) *Lipids.* The cells of the adrenal cortex are characterized by the high concentration and labile nature of cytoplasmic lipid. A number of dyes are soluble in tissue lipids. None of them selectively combines with any single lipid or class of lipids. Kaufmann and Lehmann (225) have shown that Sudan stains free fatty acids, triglycerides of fatty acids, cholesterol esters and free and combined lecithin. According to the *Microtomist's Vade-Mecum* (133), Sudan and Scharlach R stain fatty acid esters and cholesterol esters, and Mallory (279) is of the opinion that Sudan stains almost all fat occurring in the body. The relative intensity and distribution of lipid in the adrenal cortex are independent of the stain employed. Osmic acid, a substance which reacts with many non-fatty substances (133, 279), is an exception to this rule. In the reviewer's experience, a very close parallelism exists between the density of sudanophilic material and the concentration of total cholesterol in the adrenals, including circumstances in which these substances are stored in excess, depleted or present in normal quantities.

Marked fluctuations in the intensity of lipid staining of the adrenal cortex occur under a variety of circumstances. The literature on this subject has been reviewed and interpreted in the light of changes in lipid staining following the administration of ACTH (399). In general, lipid depletion denotes hyperactivity and lipid accumulation hypoactivity of the gland. However, Deane *et al.* (79) describe two types of lipid depletion of the adrenal cortex; lipid depletion associated with the presence of small cells characterizes a gland which has been hypoactive for an extended period of time, whereas lipid depletion in association with large cells denotes hyperactivity.

According to Sayers and Sayers (396), the changes in the intensity of both sudanophilic staining and cholesterol concentration of the adrenal cortex are mediated by ACTH. These investigators found a close parallelism between the intensity of sudanophilic substance and the concentration of cholesterol in the gland. However, Skelton *et al.* (436) claim that under certain circumstances intensity of Sudan staining and cholesterol concentration do not run in parallel. The point is of practical as well as theoretical importance and deserves further experimental study. In the reviewer's experience, considerable variation in intensity of Sudan staining may result from slight variations in technic. For this reason, it is neither as reliable nor as quantitative an index of adrenocortical activity as the chemical analysis of cholesterol concentration.

b) *Localization of secretory zones.* Histochemical technics have a distinct advantage over chemical analyses in their ability to distinguish the various zones of the adrenal cortex. The glomerular, sudanophobic, fascicular and reticular zones may show different degrees of depletion and accumulation of lipid in response to stress. Yoffey and Baxter (514), using a variety of staining technics, concluded that the changes in the glomerulosa are independent of those in the remainder of the cortex. Ducommun and Mach (103) have shown that the histochemical changes produced by ACTH are less marked in the glomerulosa than in the other zones of the adrenal cortex. These differences in response are part of the evidence which has prompted some investigators to assign specific secretory activities to the individual zones, a subject which will be discussed in detail in another section.

c) '*Ketosteroids.*' Histochemists are not yet in a position to identify or localize

the steroids of the adrenal cortex. However, Dempsey (83) and Deane *et al.* (79) continue to describe the 'ketosteroids' of the adrenal cortex, despite the overwhelming amount of evidence that the stains which they employ reveal neither active nor inactive ketosteroids in the gland. According to Dempsey (83), no substance other than those which belong to the ketosteroid group is presently known which exhibits all five of the following reactions: *1*) acetone solubility, *2*) reactivity with carbonyl reagents (phenylhydrazine, Feulgen's leucofuchsin), *3*) autofluorescence, *4*) birefringence, and *5*) reactivity with concentrated sulfuric acid, as in the Liebermann-Burchard reaction.

Gomori (146), confirmed by Claesson and Hillarp (55), has clearly demonstrated that fresh adrenal tissue will react visibly with neither Schiff's reagent nor phenylhydrazine; it is only after oxidation, as occurs with fixation, that these reagents react with substances in the tissue. Albert and Leblond (3) have shown that removal of all known sources of ketosteroids (adrenals and gonads) does not modify the phenylhydrazine and leucofuchsin reactions in a number of tissues. Furthermore, there is a strict parallelism between the actions of phenylhydrazine and Feulgen's leucofuchsin reagent in all tissues; both reactions are intensified by mercuric bichloride, a fact strongly suggesting that it is tissue plasmalogens, which liberate fatty acid aldehydes upon hydrolysis, and not ketosteroids which are the protoplasmic reactants for these stains.

Boscott and Mandl (35) found that dinitrophenylhydrazine does not react with adrenal tissue when aldehydes are first eliminated by condensation with aromatic amines; steroid ketones apparently are not responsible for the positive dinitrophenylhydrazine reaction of adrenocortical tissue. Claesson and Hillarp (55) came to the conclusion that the birefringent, digitonin-precipitable substance in the ovary is not a ketosteroid substance but most likely cholesterol. These same workers demonstrated that the greenish fluorescence of the ovary and adrenal cortex is ultraviolet labile and hence is not ketosteroid material but probably vitamin A. The complexity of lipids in tissues, in particular the adrenal cortex, makes it extremely difficult to identify substances on the basis of staining properties. Finally, the concentration of active cortical steroids in the adrenal gland is extremely minute and it is difficult to see how even specific stains could make such traces of material visible.

Ashbel and Seligman (16) have devised a new technic for staining tissue aldehydes and ketones; these functional groups are reacted with 2-hydroxy, 3-naphthoic acid hydrazide, followed by coupling of tetrazotized diorthoanisidine into the naphtholic ring with the production of a blue, insoluble azo compound. The reaction has demonstrated the presence of carbonyl-containing substances in formalin-fixed adrenal tissue but not in fresh adrenal tissue (411). Advantages of this technic over the phenylhydrazine method are not striking. The localization of carbonyl groups in tissue is similar to that previously demonstrated with other methods. In addition, nonlipid material in tissues gives a blue color with the stain.

The battery of specialized staining technics which has been applied to the adrenal has contributed little more to our knowledge of the chemistry and physiology of the adrenal cortex than has the simple Sudan method. It is recognized that the amount of lipid present, as measured by intensity of Sudan stain, may not parallel

materials exhibiting 'steroid reactions' (birefringence, fluorescence, Schiff reaction) (15, 24, 154). However, until more is known about the chemical nature of the latter materials it is wise to withhold physiological interpretations. Statements regarding storage and secretion of 'ketosteroids' have contributed nothing but confusion to the field.

3) CHEMICAL. Fluctuations in the secretory activity of the adrenal cortex are associated with readily measurable changes in the concentration of certain chemical constituents in the gland.

a) *Cholesterol.* Cholesterol is present in high concentration in the adrenal cortex. When the gland is relatively inactive, approximately 5 per cent of the wet weight of the tissue is cholesterol, a concentration higher than that found in any other tissue of the body, including brain. The cholesterol content of the adrenal is usually determined by the Sperry-Schoenheimer method (439). The technic does not measure 'total steroids' of the adrenal cortex, as believed by Bergner and Deane (28); rather it measures those steroids with a hydroxyl group in the beta configuration at position 3 of the cyclopentaphenanthrene nucleus and which give a color with the Liebermann-Burchard reagent. Biologically active cortical steroids are not included because they have a carbonyl group at carbon 3 and are therefore not precipitated by digitonin. The bulk of the reacting material is very likely cholesterol, although the reviewer is unaware of isolation studies designed to determine with more certainty the exact nature of the steroid substance or substances in the adrenal which are measured by the Sperry-Schoenheimer technic.

The characteristic labile nature of adrenal cholesterol is in contrast to the relatively fixed concentration of the steroid in other tissues. Situations which may be expected to increase the secretory activity of the adrenal cortex are associated with a reduction in the concentration of cholesterol in the adrenal. The extensive literature on this subject has been reviewed (399) and correlated with the fact that ACTH depletes the cholesterol content of the adrenal cortex at a time when metabolic changes characteristic of adrenocortical hyperactivity are occurring in the organism (139, 396, 399). In the absence of the anterior pituitary, adrenal cholesterol is inert to stress and usually fixed at a concentration higher than normal (339, 400). These physiological phenomena, together with the fact that cholesterol is closely related chemically to the cortical steroids, strongly suggest that cholesterol is a precursor of the cortical hormone. The large store of cholesterol in the gland appears to be held in readiness for periods of acceleration of cortical hormone synthesis; cortical hormone itself is not stored in the gland in any significant amount. More direct evidence is available that cholesterol is a precursor of progesterone (32), a substance closely related chemically to the cortical steroids. Organic chemists have been unwilling to accept cholesterol as a precursor of cortical hormone because it is difficult in the test tube to introduce oxygen atoms into positions 11 and 17 of the cyclopentaphenanthrene nucleus. However, since it has been demonstrated that the perfused adrenal can introduce an oxygen at C-11 (173), the objection may be ignored.

The adrenals of an adult rat, kept under optimal environmental conditions, contain 2 to 3 mg. of cholesterol. Stress (400) and ACTH (401) can deplete the content of cholesterol in the adrenal by 50 per cent in three hours, a disappearance of one

to 1.5 mg. of the sterol. Quantitative conversion of this amount of cholesterol to active cortical steroids would give an amount of the latter in the range of present estimates of rate of cortical hormone production (table 2). The circulating blood may contribute to the stores of adrenal cholesterol; furthermore, the recent report by Srere *et al.* (442) that cholesterol is synthesized by adrenocortical tissue *in vitro* indicates that the above estimate; based on stored material alone, is a minimal value.

A number of investigators have employed alterations in the cholesterol content of the adrenal to determine the time course and intensity of adrenocortical activity during the response of the organism to a variety of stresses (see section on classification of the responses of the adrenal cortex to stress). There appears to be a good inverse correlation between the concentration of adrenal cholesterol and the secretory activity of the gland as predicted by the intensity and duration of the stimulus. Cholesterol analysis may be a valuable supplement to gland weight in estimating adrenocortical activity. Unfortunately, studies have not yet been initiated to determine whether a quantitative relationship exists between the dose of injected ACTH and the degree of reduction in adrenal cholesterol.

The amount of adrenal cholesterol may be influenced by factors other than the adrenocorticotrophic activity of the pituitary. Care must be employed in interpreting the results of adrenal cholesterol analyses in debilitated animals or man until such time as a better understanding is gained of the factors which influence rate of synthesis and accumulation of cholesterol in the gland.

b) *Ascorbic acid.* The extensive literature on the alterations in the concentration of ascorbic acid in the adrenal under a variety of experimental conditions has been reviewed (396). In general, increased adrenocortical activity is associated with a reduction in the concentration of adrenal ascorbic acid. Administration of ACTH rapidly depletes adrenal ascorbic acid in the rat and guinea pig (401); the concentration of adrenal ascorbic acid falls appreciably within 20 minutes after injection of the hormone into rats (401). The regulatory control of the adenohypophysis is further demonstrated by the fact that, in its absence, adrenal ascorbic acid is unaffected by stress (400).

The role of ascorbic acid in the metabolic processes of the adrenal is unknown. Lowenstein and Zwemer (271) claimed that a glycoside of a cortical steroid, extracted from adrenocortical tissue with chloroform, could be split by mild hydrolysis. In addition, these same workers (271) reported the isolation and characterization of a water-soluble conjugate of a cortical steroid and ascorbic acid. The observations have not been confirmed. If Lowenstein and Zwemer are correct, then an increase in the secretory activity of the adrenal would be expected to increase the concentration of ascorbic acid in the effluent blood of the gland. Vogt (489) has found no consistent differences between the ascorbic acid contents of the blood entering and that leaving the adrenal cortex under conditions of increased activity; neither could she find detectable quantities of ascorbic acid in ethylene dichloride extracts of adrenal vein plasma.

A number of physiological studies suggest that ascorbic acid plays an important role in the secretory activity of the adrenal cortex. Lockwood and Hartman (265) were impressed by the similarity between the symptoms of adrenocortical insuffi-

ciency and of scurvy. According to these investigators adrenocortical extract (ACE), free from ascorbic acid, retarded the onset of symptoms and decreased the body weight loss of vitamin C-deficient guinea pigs. Ratsimamanga (368) claimed that adrenocortical extract prolonged the survival of scorbutic guinea pigs. Likewise Kendall (cited as a personal communication in (312)) noted that scorbutic animals, given adrenocortical extract, survived longer than those not treated, although the extract did not cure the scurvy since the pathological lesions at death were the same in both groups. Like the adrenalectomized animal, the scorbutic guinea pig is hypersensitive to stress (334) and has a reduced natural resistance to infection (353, 354). Furthermore, there is a marked increase in the rate of utilization and/or destruction of ascorbic acid in man following burns or fractures (14, 22, 276), conditions expected to be associated with increased adrenocortical activity.

Contrariwise, an equally impressive list of observations suggests that ascorbic acid does not play an essential role in the functional activity of the adrenal cortex. Impairment of deposition of liver glycogen in scorbutic animals, a defect in carbohydrate metabolism characteristic of scurvy, is not improved by treatment with adrenocortical extract (292, 312). Patients with scurvy excrete corticoids, as determined chemically, at a rate within the normal range (75). *Plasmodium knowlesi* infection induces marked decreases in the concentration of adrenal ascorbic acid in monkeys (291), but *Plasmodium gallinaceum* has no effect on the concentration of the vitamin in the chick adrenal (223). Furthermore, neither epinephrine nor ACTH influences the concentration of ascorbic acid in the chicken adrenal (34). Finally, ACTH produces the usual fall in adrenal cholesterol, together with a lymphopenia, in guinea pigs in whom the concentration of ascorbic acid in the adrenal has been reduced practically to zero by a scorbutic diet (268). Ascorbic acid does not appear to be an essential component of those metabolic processes of the adrenal concerned with the secretion of cortical hormone. It is possible that some other constituent of the gland, e.g., glutathione, can substitute for the vitamin.

In general, there is a parallelism between the concentrations of cholesterol and ascorbic acid in the adrenals. However, certain important exceptions exist. Following hypophysectomy the concentration of cholesterol in the adrenal remains fixed or increases (399), whereas the concentration of ascorbic acid in the gland decreases (475). The cholesterol content of the adrenal of guinea pigs on a scorbutic diet may be normal or above normal at a time when the ascorbic acid content is reduced practically to zero (268). In rats infected with *B. tularense*, normal levels of adrenal ascorbic acid may be associated with extremely low cholesterol concentrations (357). Exposure to low atmospheric pressure for five hours has been reported to produce a marked reduction in the concentration of adrenal cholesterol but only slight change in the ascorbic acid content of the gland (461). In the rat, immediately following the administration of ACTH or the application of stress, the adrenal is more rapidly depleted of ascorbic acid than of cholesterol. During the recovery phase, the pre-stress level of adrenal ascorbic acid is attained much more quickly than that of cholesterol. In the guinea pig the rates of recovery of the two substances are approximately equal (401).

Levels of adrenal ascorbic acid must be employed with caution as indices of

adrenocortical activity until a better understanding is gained of the role of ascorbic acid in the metabolic processes of the adrenal cortex. The level of ascorbic acid in the adrenal at any instant is undoubtedly determined by at least three factors, namely, rate of utilization, rate of discharge, and rate of synthesis; therefore, a number of variables other than the rate of secretion of the cortical steroids may be expected to exert a modifying influence. In the reviewer's experience, adrenal ascorbic acid is a very reliable index of adrenocortical activity in acute experiments in healthy rats. However, in acute stress in animals with nutritional deficiencies or in chronic experiments involving malnutrition or prolonged stress, factors other than the rate of secretory activity of the adrenal cortex play a modifying and sometimes even a dominant role in determining the level of the substance in the gland. For instance, equal degrees of stress will produce a greater reduction of adrenal ascorbic acid in a malnourished than in a normal animal. It is unlikely that the adrenal cortex is more active in the deficient animal; it is more reasonable to suppose that the lower post-stress level of adrenal ascorbic acid in the deficient animal is a result of interference with synthesis of the vitamin. Pinchot *et al.* (357) have come to the conclusion that ascorbic acid concentration "—alone is not a reliable index of the functional state of the adrenal which has been subjected to a prolonged stress."

c) *Phosphorus.* Administration of ACTH in the rat increases the rate of turnover of radioactive phosphorus in the adrenal cortex (139). The potential usefulness of radioactive phosphorus as an index of adrenocortical activity remains to be determined; additional studies are awaited with interest.

II. *Alterations in the Organism.* 1) LYMPHOCYTES. The observation of Dougherty and White (102) that circulating lymphocytes are under the regulatory control of the adrenal cortex has made available a valuable tool for the measurement of the activity of the adrenal cortex in man and experimental animals.

The lymphocytopenia that results from a wide variety of unrelated stimuli is due to increased activity of the adrenal cortex. The phenomenon is initiated by the discharge of ACTH from the adenohypophysis (100, 102) and is induced by those cortical steroids with an oxygen on carbon 11 (17, 100, 102, 124, 180). It has been reported that neither stress nor ACTH will induce a lymphocytopenia in the adrenalectomized animal (100, 102, 112, 113, 278, 374) or in the patient with Addison's disease (17, 124, 180). Presumably, the lymphocytosis of the adrenalectomized animal (62, 100, 102, 113, 519) and of the patient with Addison's disease (17, 81, 131) reflects the low blood titer of adrenocortical hormone in these conditions. Conversely, the lymphocytopenia of Cushing's syndrome (81) would appear to be a result of an elevated titer of cortical hormone.

The simplicity of the technic for measuring circulating lymphocytes has prompted a number of workers to employ this index as a measure of adrenocortical activity in man under a variety of circumstances. Pain (280) and infusion of epinephrine (185) induce a lymphocytopenia in man. Inability to induce a lymphocytopenia with ACTH may be taken as an indication of adrenocortical deficiency (17, 124, 180). Psychotic men fail to respond with a lymphocytopenia to the stress of heat (359) or of operating a pursuit meter under conditions of anoxia (181); it has been concluded that these individuals have a certain degree of inertia of the pituitary-adrenal system.

The number of lymphocytes circulating in the blood at any moment is determined by a number of factors, among which the adrenal cortex is an important but not a sole factor. Circulating lymphocytes can be employed as an index of adrenocortical function only with certain precautions. A few agents, e.g. x-irradiation (101) and urethane (106), have a direct lymphocytolytic action as shown by the lymphocytopenia they produce in adrenalectomized animals. Lewis and Page (253) conclude that the lymphocytopenia elicited by typhoid vaccine is not dependent on the discharge of adrenocortical hormone since it produces a lymphocytopenia in adrenalectomized rats. The specificity of the index is not absolute in man, for it has been reported that epinephrine (0.75 mg.) (131) and glucose administration (219) induce a lymphocytopenia in patients with Addison's disease. Splenic discharge of lymphocytes may obscure the lymphocytopenia of stress under certain conditions (171, 447); this probably is the reason why the lymphocytopenia following epinephrine infusion in man is preceded by a lymphocytosis (185).

Epinephrine produces a lymphocytopenia in the hypophysectomized but not in the adrenalectomized rat, although the response in the absence of the pituitary is much less than normal (189). Does this mean that epinephrine stimulates the secretion of the cortical steroids by a direct action on the adrenals? The hypophysectomized animal has a small but nevertheless a definite quantity of cortical hormone released continuously by the adrenal cortex. Can epinephrine act directly on lymphocytes in the presence of this small titer of hormone? This raises the question as to whether the adrenal cortex is an inciting or a supportive agent in the lymphocytopenia of stress. Is an actual increase in the secretory activity of the adrenal cortex a necessary step in the lymphocytopenia of stress, or can stress induce a lymphocytopenia in an adrenalectomized animal given maintenance quantities of C-11 oxygenated steroids? It is to be hoped that experimental studies designed to settle this problem are forthcoming.

The discussion has been confined to the lymphocytopenia of acute stresses, i.e., situations in which the noxious stimulus acts for a few hours. It has been reported that daily administration of ACTH for 15 days induces a lymphopenia in mice (500). However, in chronic stress a complexity of factors may influence the number of circulating lymphocytes and obscure the contribution of the adrenal cortex. Infectious diseases are very often accompanied by a marked lymphocytosis (180) despite the increased activity of the adrenal cortex in response to the stress of fever and toxins.

In experimental animals, the total mass of lymphoid tissue varies inversely with the activity of the adrenal cortex (99). Adrenal hypertrophy and ascorbic acid depletion accompany thymic involution in mice bearing sarcoma 180 tumors (391). However, it is to be emphasized that the adrenal cortex is only one of a number of factors influencing the total mass of lymphoid tissue, and this index must therefore be applied with certain limitations in mind. For example, urethane and methyl-bis-(β-chlorethyl)-amine have a direct lytic action on lymphoid tissue (19), and the thyroid hormone induces an increase in lymphoid tissue mass in adrenalectomized mice (281).

There appears to be a lack of parallelism between lymphoid tissue mass and other indices of adrenocortical function during fasting. A fast of 96 hours' duration in the mouse produces a shrinkage of lymphatic tissue mass to one half of normal without

producing a change in adrenal weight (97). The reviewer has found little change in the weight, chemistry or histochemistry of the adrenal of the rat after three to five days of fasting, despite a marked decrease in thymus weight. Total mass of lymphoid tissue may be employed as an adjunct to other measures of adrenocortical activity, but it is not of consequence when used alone. In conclusion, it may be stated that the determination of the number of circulating lymphocytes has proven to be a valuable tool in the evaluation of adrenocortical activity, particularly in species in which the lymphocytes constitute a major fraction of the total white blood cell count. It has the considerable advantage that measurements can be made without killing the animal. However, the method is not without limitations and, like other indices, due account must be taken of certain factors other than the adrenal cortex which may interfere.

2) Eosinophils. The observation of Hills *et al.* (180) that the number of circulating eosinophils is determined by the activity of the pituitary-adrenocortica system has made available a very simple method for assessing adrenocortical func-l tion. The eosinophil test appears to be the most useful index in man. Following the administration of ACTH, the drop in the number of eosinophils, expressed in percentage of the pre-injection count, is approximately twice as great as the drop in the number of lymphocytes; furthermore, the eosinophil response is less variable than the lymphocyte response (124).

Stresses such as labor (76), eclampsia (76), surgery (8, 76), administration of epinephrine (8, 141, 185), injection of insulin (8, 141), and electroconvulsive therapy (10) produce an eosinopenia in man. Hills *et al.* (180) state that "—a wealth of reports in the literature attests the great variety of clinical emergencies in which a striking diminution of the eosinophils is found." The interested reader may refer to the article by Hills *et al.* (180) for an enumeration of these various stresses.

In man, absence of an eosinopenia following the administration of ACTH is indicative of adrenocortical insufficiency (468). When surgery is not followed by an eosinopenia, failure of the adrenal cortex should be suspected (381). The measure promises to be the first practical test of the functional capacity of the adrenal cortex in emergency shock-like states; heretofore, the decision to employ cortical hormone therapy in such states has been made in most instances without rational basis.

Continued exposure of man to extremes of environmental temperature is accompanied by a persistent depression of the number of circulating eosinophils (443). On the other hand, no significant changes occur in 17-ketosteroid excretion, urinary uric acid to creatinine ratio or number of circulating lymphocytes. Additional studies on the value of circulating eosinophils as an index of adrenocortical activity in chronic stress in man are awaited with interest.

The eosinophil test, like the lymphocyte test, is of undoubted value in the diagnosis of complete or nearly complete adrenocortical insufficiency. However, the value of the test for the assessment of partial impairment of adrenocortical function must await the accumulation of additional experimental data. Speirs and Meyer (438) have reached the tentative conclusion that the eosinophil test will detect relative adrenocortical insufficiency in mice.

Eosinophils are influenced by factors other than the secretion of the adrenal

cortex. Considerable caution must therefore be exercised in the interpretation of eosinophil changes, particularly when the organism is subjected to a severe degree of stress. The blood picture of patients with Addison's disease remains fixed under most conditions, but an eosinopenia can occur in infectious diseases in these individuals (17). Furthermore, large doses of epinephrine induce an eosinopenia even in the absence of the adrenal cortex. Thorn *et al.* (467) make the following statement: "Following the administration of 0.2 mg. of epinephrine hydrochloride, an intact anterior pituitary gland will release sufficient ACTH to stimulate a normal adrenal cortex to increase the secretion of steroid hormones. Adrenal steroids identical with or related to 11-17-hydroxycorticosterone will lead to a rapid fall in circulating eosinophils which will reach a maximum in approximately four hours after the beginning of the injection. In the absence of adequate anterior pituitary or adrenocortical function this change will not occur." Recant *et al.* (370) state: "—1.5 mgm. of epinephrine in saline given intravenously over a one-hour period produces a fall in circulating eosinophils of from 55 to 75 per cent in four hours in both normal subjects and *patients with Addison's disease.*" (Italics are the reviewer's.)

It is obvious that in evaluating the functional capacity of the pituitary-adrenocortical system attention must be paid to the test dose of epinephrine. Perhaps the instances in which epinephrine has been reported to have a lymphopenic action in Addison's disease are those in which relatively large doses of epinephrine have been employed. However, the possibility that positive responses have occurred in patients with Addison's disease because of a relative deficiency rather than an absolute loss of function must also be considered. Additional studies in which the eosinophil test is run in parallel with other indices of adrenocortical function are necessary before the limitations of the technic will be completely understood. At any rate, the eosinophil test is the most simple and practical measure of adrenocortical activity in man. The test has already proven of great value in numerous studies designed to correlate the adrenocortical stimulatory action of ACTH with its therapeutic action.

3) METABOLIC. Adrenocortical insufficiency is characterized by several metabolic changes including insulin hypersensitivity, lack of diuresis after administration of water, and failure to retain sodium under circumstances of sodium deprivation. The demonstration of these metabolic defects is supportive evidence for the diagnosis of Addison's disease in man; the procedures employed for their detection are presented in detail in the monograph by Thorn *et al.* (467). The tests are of no value in determining the degree of adrenocortical activity in an organism with an intact pituitary-adrenocortical system, for at least two reasons. First, stresses induce metabolic aberrations which obscure or mask those directly related to the adrenal cortex. Second, the cortical hormone, as will be considered in more detail ln a iater section of this report, has a diversity of actions depending upon the state of the organism, e.g., the hormone may be anabolic or catabolic, and may induce retention or excretion of sodium.

ACTH (468) and electrically-induced convulsions (9) increase the rate of excretion of uric acid in man. Failure of the ratio of urinary uric acid to creatinine to rise after ACTH administration is indicative of adrenocortical insufficiency (468).

III. *Urinary Steroids.* Substances are present in human urine which exhibit

certain properties of the cortical hormone. Assays of corticoids are conducted on crude concentrates of urine, hence the name urinary 'corticoids'—substances in urine which have not been clearly identified but which have one or more of the characteristics of the cortical hormones. Corticoids which have similar biological properties and which are determined by bioassay may be designated 'biocorticoids' and those which have similar chemical properties and which are determined by chemical assay may be designated 'chemocorticoids.' Obviously, the two groups overlap considerably.

1) Urinary Biocorticoids. When injected into adrenalectomized animals, ethylene dichloride extracts of human urine have been shown to exert biological actions characteristic of the cortical hormone, namely, promote deposition of glycogen in the liver (92, 186, 481, 483), maintain life (89, 481), prolong survival from exposure to cold (90, 94, 481, 495), improve work performance (432), and prevent water intoxication (405). The recent isolation of cortisone (283, 406) and 17-hydroxycorticosterone (283) from human urine gives additional support to the belief that the biologically active substances in the crude extracts are cortical steroids.

The properties of cold-protection (CP) and liver-glycogen deposition (LGD) have been made the basis of quantitative assays of urinary biocorticoids. The LGD test is less sensitive but more accurate than the CP test (93, 94, 481). Cortical steroids with an oxygen on carbon 11 of the steroid nucleus are responsible for cold-protection and liver-glycogen deposition; desoxycorticosterone exerts either no influence or only a minor one. It is unlikely that a satisfactory bioassay method will be devised for the determination of so-called 'salt-active' desoxycorticosterone-like steroids of urine since 11,17-oxysteroids (cortisone-like steroids) are not without influence on electrolyte metabolism.

Results of urinary biocorticoid analyses are presented in table 1. There is a close correlation between the rate of biocorticoid (LGD) excretion and the activity of the adrenal cortex as judged by clinical criteria. However, malnutrition appears to influence the rate of excretion of biocorticoids (table 1). The possibility has been entertained that malnutrition interferes with the functional capacity of the pituitary-adrenal system; however, evidence from animal experiments, which will be discussed in greater detail in a later section, fails to support this explanation.

Biocorticoid excretion is a relatively good index of adrenocortical activity; unfortunately, the method of assay is tedious and time-consuming and not practical in most clinical laboratories.

2) Urinary Chemocorticoids. The assay technics take advantage of the solubility as well as the chemical characteristics of the cortical steroids. For example, in the method of Heard *et al.* (170) the urine is extracted with an ether-chloroform mixture. The ether-chloroform extract is washed with aqueous alkali to remove acids and phenols (phenolic estrogens). Analysis of chemocorticoids may be made on this neutral-lipid extract of urine (170) or after fractionation of the crude concentrate between water and benzene (456), which separates compounds such as 17-hydroxycorticosterone and cortisone (aqueous phase) from less water-soluble compounds similar in character to desoxycorticosterone. Further fractionation into ketonic and non-ketonic substances can be made with Girard's reagent T (456). The following chemical reactions may be applied to one or more of these fractions.

a) *Phosphomolybdic acid* is reduced "— by steroids containing a primary or secondary (but not tertiary) α-ketol function, an α,β-unsaturated 3-ketone group, or both" (169). Desoxycorticosterone, cortisone, progesterone and a large number of other biologically active as well as inactive steroids will reduce phosphomolybdic acid (169).

b) *Copper* is reduced by steroids having an α-ketol function. An α,β-unsaturated 3-ketone group does not react, hence progesterone does not interfere with this test (169).

c) *Formaldehyde* is generated by the reaction of steroids with periodic acid when the steroids contain α-ketol or glycol side chains. The method, first described by Lowenstein *et al.* (270), was based on earlier studies by Fieser *et al.* (120). The method has been improved, and instead of treating the urine concentrate directly with chromogenic agent the formaldehyde is distilled off and color developed in the distillate (61, 74).

Lipids that reduce phosphomolybdic acid may be designated PRL; those that reduce copper, CRL; and those that generate formaldehyde, FL (385).

It is to be emphasized that these chemical reactions are not specific for cortical steroids, but are entered into by a large number of organic compounds which are normally present in blood and tissue. The relative freedom of urine from lipid material together with the nature of the method of extraction of the urine largely determines the degree of specificity obtained. Both blood and feces, for example, contain lipid-soluble reducing substances which seriously interfere with the determination of chemocorticoids. Urine contaminated with blood or feces gives false values (170) Hemphill and Reiss (174) have reported chemocorticoid analyses of adrenocortical tissue and blood and Nichols and Miller (319) have published analyses of sweat without cognizance of these important facts.

The results of numerous analyses of chemocorticoids in urine are presented in table 1. First, it is to be noted that the chemocorticoids are present in higher concentration in urine than are the biocorticoids, i.e., biocorticoids determined by liver glycogen deposition and expressed as equivalents of cortisone. Biologically inert substances having certain of the chemical properties of the cortical steroids may arise as a result of the degradation of the active compounds in the liver and other tissues. Desoxycorticosterone, if present in urine, would be included in the chemocorticoids, not in the biocorticoids.

Second, it is to be noted that there is considerable variation in the absolute concentrations as determined by the various methods of analysis. At least two variables are responsible, the method of extraction and the chemical reaction. However, it is obvious from the data presented in table 1 and in particular from those of Romanoff *et al.* (385), who made careful comparative studies, that there is a close correlation among the results of the three methods in regard to relative amounts. Furthermore, Romanoff *et al.* (385) came to the conclusion that the benzene:water partition offers "no special advantage since the ratio between the titers of two incident fractions shows no special alteration with either diurnal stress or ACTH administration."

Third, according to Matson and Longwell (286), both premature and full-term

TABLE 1. URINARY CORTICOIDS AS DETERMINED BY BIOLOGICAL AND CHEMICAL METHODS

METHOD; INVESTIGATOR; REFERENCE STANDARD	CONDITION	URINARY CORTICOID EXCRETION
		mg/day
Biocorticoids		
Liver glycogen deposition (LGD); Venning *et al.* (483), Venning and Browne (480), Venning and Kazmin (482), Venning *et al.* (484); cortisone	Normal adult males (14)[1]	0.060 (0.040–0.085)
	Normal adult females (14)	0.041 (0.025–0.065)
	New-born, first 10 days of life (17)	0.003–0.017
	Normal males 2½–3 yr. (3)	0.035, 0.037, 0.040
	Normal male 5½ yr. (1)	0.055
	Addison's disease (4)	0.015, not detectable in 3 cases
	Panhypopituitarism (5)	0.005, 0.010, not detectable in 3 cases
	Anorexia nervosa (3)	0.020, 0.020, 0.033
	Cushing's syndrome (5)	0.040, 0.14, 0.28, 0.7, 0.7
	Diabetes (2)	0.045, 0.055
	Normal males, muscular exercise (pool of 14 subjects)	0.160 (1st expt.)
		0.210 (2nd expt.)
	Trauma in well-nourished subjects	Generally 0.2–0.3, as high as 0.4
	Trauma in malnourished subjects	Rise usually less than normal
	Pregnancy	Slight rise during first trimester, decline to normal then sharp rise beginning middle of pregnancy (over 0.3 in some cases) with falling-off before parturition
LGD; Shipley *et al.* (431); 11-dehydrocorticosterone	Normal females (5)	<0.5, 0.8, 0.5, <0.2, <0.2
	Normal males (7)	<0.3, <0.3, 0.5, 0.4, <0.2, <0.5, 0.3
	Infectious disease, active phase (6)	1.8, 2.1, 0.5, 2.0, 0.4, 0.6
	Surgery (1)	4.9
	Burns (1)	3.7
LGD; Selye (412); cortisone	Hypertensive males (9)	0.053, 0.117, 0.201, 0.184, 0.091, 0.134, 0.058, 0.043, 0.072
	Hypertensive females (8)	0.082, 0.045, 0.045, 0.051, 0.053, 0.063, 0.042, 0.126
Cold-protection (CP); Shipley *et al.* (431); 11-dehydrocorticosterone	Normal females (2)	1.4, 1.5
	Normal males (5)	0.7, 0.5, 1.0, 1.8, 1.0
	Infectious disease, active phase (2)	1.8, 3.6
	Surgery (1)	3.0
	Burns (1)	3.7
Chemocorticoids		
Copper-reducing lipid (CRL) (benzene-water partition); Talbot *et al.* (455, 456); corticosterone	Normal adults (9)	0.24 (0.10–0.38)
	Addison's disease (6)	0.12 (0.02–0.26)
	Hypopituitarism (3)	0.14 (0.10–0.17)
	Cushing's syndrome (3)	4.20 (0.90–12.0)
	Adrenal cortical virilism (2)	0.43 (0.15–0.57)
	Burn and postoperative patients (4)	0.93 (0.34–1.70)
CRL (no benzene-water partition); Tompsett and Oastler (473); 11-desoxycorticosterone	Normal subjects (4)	0.10–0.16
	Simple obesity (4)	0.12–0.20
	Obesity with essential hypertension (2)	0.36, 0.53
	Essential hypertension (6)	0.16–0.25
	Diabetes mellitus (2)	0.17, 0.20
	Panhypopituitarism (1)	0.13
	Cushing's syndrome (2)	1.21, 0.88
	Adrenogenital syndrome (1)	0.32
	Burns (6)	0.20, 0.34, 0.90, 0.99, 1.73, 2.52
	Surgery (6)	0.62, 0.62, 0.53, 0.89, 1.23, 0.18
CRL; Forsham *et al.* (124), (analyses by Talbot); corticosterone	Normal male (1), Control	0.22
	ACTH 40 mg/day	1.34, 0.71
	Male pituitary deficiency (1), Control	0.32, 0.36
	ACTH 40 mg/day	1.50, 1.65
	Female asthenia (1), Control	0.60
	ACTH 40 mg/day	1.35, 2.42
	Female Addison's disease (1), Control	0.12
	ACTH 40 mg/day	0.10

TABLE 1—(*Continued*)

METHOD; INVESTIGATOR; REFERENCE STANDARD	CONDITION	URINARY CORTICOID EXCRETION
		mg/day
Chemocorticoids		
Phosphomolybdic acid-reducing lipids (PRL) and LGD (no benzene-water partition); Heard and Sobel (169), Heard *et al.* (170); 11-desoxycorticosterone (PRL), cortisone (LGD)		PRL LGD
	Normal adult males (9)	1.53 (1.1–2.1) 0.062 (0.045–0.077)
	Normal adult females (9)	1.34 (1.0–2.0) 0.039 (0.025–0.055)
	Panhypopituitarism (2)	0.46 0
		0.62 0
	Cushing's syndrome (1)	4.80 0.212
	Pregnancy (2)	2.48 0.111
		2.63 0.125
	Burns (3)	3.0 0.121
		3.4 0.180
		2.7 0.102
PRL (no benzene-water partition); Matson and Longwell (286); 11-desoxycorticosterone	Full-term infants (5)	0.35 (0.26–0.62)
	Premature infants (8)	0.27 (0.17–0.38)
Formaldehydrogenic lipids (FL) (no benzene-water partition); Lowenstein *et al.* (270); 11-dehydrocorticosterone	Normal males (?)	0.5–0.8
	Addison's disease (1)	0.15
	Cushing's syndrome (1)	21.0
FL (no benzene-water partition); Corcoran and Page (61); 11-desoxycorticosterone	Normal males (10)	1.15 ± 0.32
	Normal females (10)	0.84 ± 0.29
	Malignant hypertension (3)	0.91, 1.54, 1.74
	Addison's disease (DCA & ACE therapy) (4)	0.4, 0.4, 0.4, 0.1
	Cushing's syndrome (2)	2.7, 3.3
FL (benzene-water partition); Daughaday *et al.* (74, 75); cortin unit—a steroid, 7.6 % of which would yield formaldehyde upon oxidation with periodic acid	Normal males (6)	1.4 (1.0–1.7)
	Normal females (3)	1.0 (1.0–1.1)
	Hypertension (3)	1.0, 1.1, 1.3
	Addison's disease, Males (5)	0.5 (0.3–0.8)
	Female (1)	0.6 (0.5–0.65)
	Cushing's syndrome (3)	23.0, 2.5, 1.8
	Adrenogenital syndrome (1)	1.5
	Panhypopituitarism, Males (3)	0.5, 0.8, 0.8
	Females (4)	0.8, 0.6, 0.5, 1.2
FL (benzene-water partition, Girard's reagent T and FL (270) on ketonic fraction); Mason *et al.* (284); 11-dehydrocorticosterone	Normal female (1)	
	Av. and range 33-day control period	0.180 (0.174–0.186)
	ACTH 100 mg/day av. and range 7 days	0.955 (0.556–1.440)
PRL, FL and CRL (no benzene-water partition); Romanoff *et al.* (385); 11-desoxycorticosterone	Normal males (34)	PRL FL
	Sleep	2.11 ± 0.115[2] 0.32 ± 0.027
	Morning	2.62 ± 0.193 0.41 ± 0.026
	Day	2.51 ± 0.140 0.35 ± 0.026
	Normal females (34)	PRL FL
	Sleep	1.48 ± 0.105 0.40 ± 0.039
	Morning	1.65 ± 0.097 0.56 ± 0.045
	Day	1.75 ± 0.136 0.43 ± 0.038
	Normal females (16) and males (2)	PRL CRL
	Sleep	1.44 ± 0.132 0.49 ± 0.062
	Morning	1.71 ± 0.376 0.53 ± 0.058
	Day	1.82 ± 0.251 0.49 ± 0.075
	Normal males (3)	PRL CRL
	Control	1.62 0.66
	ACTH	2.78 0.97

[1] No. in parentheses represents no. of subjects. [2] Standard error.

infants excrete appreciable quantities of chemocorticoids (PRL). Per unit of body weight, infants actually have a high rate of excretion; adults excrete approximately 0.02 mg. per kg. per day, whereas infants excrete approximately 0.12 mg. per kg. per day. The studies of Venning *et al.* (484) indicate that the new-born also excretes greater quantities of biocorticoids (LGD) per unit of body weight than does the adult

Fourth, there is a good correlation between urinary chemocorticoids and the clinical estimate of adrenocortical activity. Despite this correlation, it is to be emphasized that in the absence of adrenocortical activity, as in Addison's disease or in the adrenalectomized dog (170), an appreciable quantity of chemocorticoids appears in the urine. It is obvious that steroids of non-adrenal origin contribute to the total quantity of chemocorticoids in urine.

The methods for determination of urinary chemocorticoids are relatively simple and suitable for routine determination in the clinical laboratory. A large number of studies on urinary chemocorticoids may be expected to appear in the literature in the near future. It is to be hoped that observations designed to evaluate the effect of nutrition and liver disease, variables which are known to influence steroid hormone metabolism, will be included in these studies.

3) Urinary 17-Ketosteroids. Relatively simple and accurate methods for the analysis of urinary 17-ketosteroids are available (see review of methods by Callow (46)); hence a large number of reports have appeared in the clinical literature concerning the rate of excretion of 17-ketosteroids under a variety of conditions of stress and disease.

In normal subjects, under optimal conditions of the environment, adrenocortical activity is responsible for two thirds of the 17-ketosteroid excretion in the male and for the total 17-ketosteroid excretion in the female. The testes contribute the remaining one third in the male. Clinical investigators (6, 30) have considered the 17-ketosteroids of the urine to be an index of the androgenic activity of the testes and the adrenal cortex. 17-Ketosteroids of urine include biologically active androgens and inactive androgen-like substances, unmodified as well as degraded products of glandular secretion. Furthermore, it is to be recalled that testosterone, a most potent androgen, is not a 17-ketosteroid. Urinary 17-ketosteroids may be end-products of androgen secretion by the adrenal gland. However, an equally plausible explanation, and one that has received experimental support in recent years, is that the cortical hormone itself is degraded in the liver and other tissues of the body to 17-ketosteroids. The following observations suggest that 17-ketosteroids and perhaps even androgens may arise as a result of the degradation of the cortical hormone. ACTH produces a parallel increase in urinary 17-ketosteroids, chemocorticoids and biocorticoids (124, 284, 394, 469, 479). The administration of cortisone is followed by an elevation in 17-ketosteroid excretion (346). Finally, Mason (282) and Lieberman *et al.* (262, 263) have isolated urinary 17-ketosteroids with an oxygen atom on C-11, a characteristic and identifying feature of the structure of the cortical steroids. *In vivo* and *in vitro* studies of the metabolism of cortical steroids by liver and other tissues will undoubtedly help clarify this important aspect of adrenocortical physiology.

Urinary 17-ketosteroid output should be rejected as an index of adrenocortical activity, not because of the uncertainty of its physiological significance but because

of the lack of correlation between the rate of 17-ketosteroid excretion and adrenocortical activity as determined by other measures.

It is true that in the absence of the adrenals in monkeys (91) and in Addison's disease in man (127, 467, 480) 17-ketosteroid excretion is markedly reduced and that performance on pursuit meters at various oxygen tensions and even routine flights increases the rate of excretion of 7-ketosteroids (358, 360). However, Forbes *et al.* (122) have shown that traumatic injuries in normal subjects induce only a brief peak rise followed by a pronounced depression in urinary ketosteroid excretion, and Venning and Browne (480) have demonstrated a decided lack of parallelism between the levels of urinary biocorticoids and 17-ketosteroids under a variety of conditions. Recent studies on conditions expected to increase adrenocortical activity throw still further doubt on the ability of 17-ketosteroid determinations to evaluate such activity. Miller *et al.* (299) found that hard physical work during four days of fasting was followed by an actual reduction in the rate of ketosteroid excretion in the urine to 31 per cent of the control level. Four days of starvation reduced the rate of 17-ketosteroid excretion in four subjects to 50 per cent of the control level (245). Whereas the rate of urinary excretion of 17-ketosteroids steadily declines with advancing age, the output of chemocorticoids remains unchanged (361). In addition to age (161), other factors including sex (127, 161), nutrition, gastrointestinal diseases, anemia, infection and particularly liver disease (127) influence the rate of excretion of 17-ketosteroids.

Forbes *et al.* (122) and Venning and Browne (480) interpret the lack of parallelism between the rate of excretion of 17-ketosteroids and of corticoids as evidence in support of a dual function of the adrenal cortex. 17-Ketosteroid excretion is taken as an index of the androgenic activity of the adrenal cortex and corticoids as an index of the rate of secretion of cortical hormone. The reduction of 17-ketosteroids with stress is taken by these investigators to mean that the androgenic activity of the gland has been reduced and that cortical hormone secretion has gained precedence to meet an emergency.

The interested reader is referred to the review of Engstrom (116) for a more detailed discussion of the significance of urinary 17-ketosteroids.

The measurement of 17-ketosteroids in the urine is of no value in assessing the rate of secretion of cortical hormone by the adrenal cortex of man. Considerable confusion has arisen in the clinical literature due to misinterpretation of the significance of urinary 17-ketosteroids. It is true that the activity of the adrenal cortex influences the rate of urinary excretion of 17-ketosteroids; but in many instances, as pointed out above, a number of variables act to mask partially or even completely the effect of adrenocortical activity.

IV. *Blood Corticoids.* 1) Blood Biocorticoids. Vogt (487) has determined the cortical hormone titer of blood by the cold-protection test. Adrenal vein blood contained measurable quantities of cortical hormone activity, whereas blood from various other vessels was inactive. Employing the liver-glycogen deposition test, Paschkis *et al.* (337) were able to demonstrate high titers of cortical hormone in blood taken from a London cannula inserted into the adrenal vein; no activity could be detected in peripheral blood. In later reports, Paschkis *et al.* (335, 336) claimed to have

detected cortical hormone in the arterial plasma of normal dogs, the concentration of which was increased by the administration of ACTH, epinephrine, formalin and insulin.

The above experiments definitely establish the fact that appreciable quantities of cortical hormone are present in adrenal vein blood. Unfortunately, the quantity in peripheral blood is not sufficient to give unequivocal responses by present bioassay technics; neither does it appear likely that any great increase in the sensitivity of bioassay methods will occur in the near future.

2) Blood Chemocorticoids. As has already been pointed out, technics for the determination of chemocorticoids in urine are not applicable to blood because of the large quantities of interfering lipids present in the latter fluid. Hemphill and Reiss (174), apparently unaware of these limitations, have applied the method of Talbot *et al.* (456) for the determination of urinary corticoids to an analysis of rat blood. Their data have little meaning in the absence of control analyses of blood of adrenalectomized animals. Corcoran and Page (61), recognizing the possibility of interfering substances in blood, have taken steps to remove phosphatides and chromogens by fractionation. Plasma samples from venous blood of normal male subjects were found to contain chemocorticoids ranging in amount from 0.11 to 0.42 mg. per 100 ml.; normal female subjects, 0.13 to 0.42; normal male dogs, 0.12 to 0.38; normal female dogs, 0.12 to 0.30. In the dog, adrenal vein plasma had much higher titers of chemocorticoids (0.88–4.8 mg./100 ml.) than peripheral blood plasma (0.33–0.93 mg./100 ml.). Epinephrine administration elevated the titers of chemocorticoids in adrenal and peripheral venous plasma. Unfortunately, Corcoran and Page did not analyze the blood of patients with Addison's disease or of adrenalectomized dogs. Their data would be much more meaningful if presented in conjunction with analyses of the blood of adrenalectomized animals subjected to stress.

A quantitative and relatively specific method for the determination of the chemocorticoids of blood would pave the way for the solution of some of the most pressing problems in adrenal physiology. It is not unreasonable to assume that developments in chemical analysis will produce such a method in the near future. Nelson *et al.* (313) have analyzed adrenal vein blood of dogs by chromatography and found that the steroid material present is probably 17-hydroxycorticosterone with a possible inter-mixture of some cortisone.

Estimates of Rate of Secretion of Adrenocortical Hormone

The rate of secretion of cortical hormone by the adrenal cortex may be determined directly by biological or chemical assay of adrenal vein blood or indirectly by estimating the quantity of cortical steroid required to replace the activity of the adrenal cortex in reproducing certain biological phenomena such as survival, growth, muscle work, etc. In making estimates by the indirect method, one assumes that administered steroid is utilized as efficiently as the endogenously secreted hormone. No correction need be made for the amount excreted since negligible quantities are eliminated in the urine.

The data obtained by a number of investigators are presented in table 2; they have been converted into the biological equivalents of cortisone. Despite the large number of variables which may enter into experiments of this nature, the estimates

TABLE 2. ESTIMATES OF RATE OF SECRETION OF CORTICAL HORMONE UNDER OPTIMAL AND STRESSFUL CONDITIONS

SPECIES AND ENDOCRINE STATUS	CONDITIONS	CRITERIA	ESTIMATE OF DAILY PRODUCTION		INVESTIGATORS AND REFERENCES
			per animal	per kg. body weight	
			mg. of cortisone[1]		
Direct Measures of Adrenal Vein Blood					
Dog, intact	Surgery	Biocorticoids (CP) in adrenal vein blood	90	9	Vogt (487)
Dog, intact	Surgery	Chemocorticoids (FL) in adrenal vein blood	20	2	Corcoran and Page (61)
Indirect Measures Based on Amounts of Cortical Hormone Required to Induce Certain Biological Phenomena					
Rat, adrenex	Optimal	Growth and survival (young growing animals)	0.03–0.06	0.6–1.2	Cartland and Kuizenga (49)
Rat, adrenex	Optimal	Growth and urinary nitrogen excretion	0.4	1.2	Ingle and Prestrud (212)
Rat, adrenex	Forced muscular work	Restoration of normal ability to perform muscular work	4	20	Ingle and Nezamis (208)
Rat, intact	Administration of ACTH	A degree of glycosuria equal to that produced by ACTH	4–5	13–17	Ingle *et al.* (205)
Rat, adrenex	Optimal I.P. glucose	Survival Prevention of collapse	0.05 1.2	0.25 6	Remington(376)
Rat, adrenex	Sea level 20,000 ft.	Survival Survival	0.1 0.6	0.5 3.0	Langley and Clarke (248)
Dog, adrenex	Optimal	Maintenance of normal level of plasma non-protein nitrogen	0.1	0.01	Cartland and Kuizenga (49)
Man, Addison's disease	Optimal	Ability to fast, EEG pattern	2.5–5	0.05–0.1	Forsham *et al.* (123)
Man, Addison's disease	Crisis	Recovery from shock of crisis	30–60	0.6–1.2	Thorn *et al.* (467)

[1] Based on the biological equivalent of beef extract (1.0 ml. of beef extract from 40 gm. of glands is equivalent to 0.2 mg. of cortisone as assayed by work test (241) and LGD test (327, 328)).

of the rate of cortical hormone secretion under optimal conditions are in remarkably good agreement. A possible exception is the quantity of cortical steroid required

to maintain a normal level of plasma non-protein nitrogen in the dog; this quantity appears to be rather low. Conclusive evidence is presented that the output of the gland is markedly increased during conditions of stress.

Estimates of Rate of 'Utilization' and Degradation of Adrenocortical Hormone

Numerous observations indicate that cortical hormone undergoes rapid inactivation in the organism. A relatively small fraction of intravenously administered ACE can be recovered in the urine. In the dog, 10 per cent was recovered, as estimated by biological activity (electrolyte balance) (167), and 3.4 per cent as determined by chemocorticoid (PRL) analysis (170). In man, 7 to 12 per cent was recovered as estimated by bioassay (CP) (481). A small fraction of injected cortisone has been recovered from the urine of patients with rheumatoid arthritis and from patients with Addison's disease (283). As pointed out above, detectable quantities of cortical hormone were found in blood from the adrenal vein, but not in blood from other vessels; neither did cortical hormone appear in peripheral blood when the liver or the liver and kidneys were removed (487).

The minute quantities of cortical hormone excreted in the urine of man under optimal or stressful conditions, as determined by either bioassay or chemical assay, lend further support to the conclusion that the cortical hormone is rapidly 'utilized' in the organism. The amount excreted by the normal male per 24 hours is equivalent in biological activity to less than 0.1 mg. of cortisone (table 1). An index to the magnitude of the daily output of the normal adrenal cortex may be obtained from the fact that absorption of 5 mg. of cortisone per day partially but not completely restores to normal the metabolic functions of the patient with Addison's disease (123). In disease or during muscular activity, the excretion of urinary corticoids may increase severalfold (table 1). However, even this amount represents a very small fraction of the quantities of hormone required to maintain eucorticism in adrenalectomized animals (194) or in patients with Addison's disease (467) exposed to comparable degrees of stress. In Cushing's syndrome urinary biocorticoids never exceed an amount equivalent to one mg. of cortisone (table 1) despite the fact that enormous quantities of cortical hormone must be elaborated by the adrenal cortex.

The conclusion appears inescapable that the cortical steroids undergo rapid change in the tissues. The nature of the change is unknown. *In vitro* studies of cortical hormone metabolism by various tissues are an experimental approach which has been neglected. Whether degradation is a necessary accompaniment of steroid hormone action or is an entirely unrelated and incidental phenomenon is a problem of fundamental importance. Until such time as these points are clarified it is well to apply the word 'utilization' of cortical steroids, without any implication as to the relation between degradation and functional activity of the hormone.

Since cortical hormone appears to be 'utilized' about as rapidly as it is secreted, the estimates of rate of secretion of cortical hormone presented in table 2 may also be used as approximations of rates of cortical hormone 'utilization.'

Nature of Secretion of the Adrenal Cortex

The exact nature of the secretion of the adrenal cortex is unknown. Present estimates regarding the number and character of steroids elaborated by the gland

are purely conjectural and are based on indirect evidence, such as types of steroids isolated from the gland after death and comparisons between the metabolic changes induced by trophic stimulation and those produced by the available crystalline cortical steroids.

Twenty-eight crystalline steroids have been isolated from adrenal tissue (372), a magnificent chemical achievement which gives little help to the physiologist in his attempts to uncover the nature of the secretion of the gland. It is certain that only minute quantities of biologically active steroids are stored in adrenal tissue. Many of the steroids isolated from the gland may represent intermediates in the synthesis of the cortical hormone or artifacts arising from the manipulations of the chemist. The recent work of Haines and associates (159) demonstrates quite clearly that the method of handling the tissue has a marked influence upon the composition of steroids in glandular extracts. Adrenal glands frozen rapidly after death of the animal yield much greater quantities of 17-hydroxycorticosterone than glands allowed to stand at room temperature for a few hours before freezing.

I. *Multisteroid Concept.* Selye (418, 422, 425), in an attempt to explain the variety of metabolic patterns which the organism may assume under different environmental conditions, has postulated the secretion of a 'glucocorticoid,' a 'mineralocorticoid,' a 'lipocorticoid' and a 'testocorticoid.' The concept implies that the secretion of the adrenal cortex may vary in composition according to the requirements of the organism. Important as the adrenal cortex is in regulating the metabolic pattern of the organism, the multisteroid concept places an undue burden upon the gland; many other organs play an equally significant role in the adjustments of the *milieu intérieur*. The diversity of patterns of metabolic change which may occur in the organism are more likely a result of the possible varieties of interactions between the numerous organs of homeostasis on the one hand and the varieties of stresses on the other, rather than a result of fluctuations in composition of the secretion of the adrenal cortex.

II. *'N' and 'S' Hormone Concept.* Albright and co-workers (5, 6, 7, 373, 455) have presented indirect and circumstantial evidence in a most convincing manner to support the view that the adrenal cortex secretes an 'S' hormone (cortisone-like compound also designated 11,17-oxysteroid) and an 'N' hormone having the anabolic properties of an androgen; the 'S' hormone is said to be secreted under the influence of an adrenocorticotrophin and the 'N' hormone under the influence of pituitary luteinizing hormone (LH).

Urinary 17-ketosteroids have been regarded by Albright and associates (5, 6, 7, 373) as an index of 'N' hormone secretion by the adrenal cortex; corticoids, as a measure of 'S' hormone secretion (6, 455). The lack of parallelism in the fluctuations of these two indices, both in normal men exposed to stress and in endocrinopathies, has been taken as evidence in support of the dual nature of the adrenocortical secretion. Administration of methyltestosterone, an androgen which is not excreted as a 17-ketosteroid, decreases the rate of excretion of 17-ketosteroids in castrate males and in females (373). Reifenstein *et al.* (373) interpret this to mean that methyltestosterone acts to suppress the release of a pituitary gonadotrophin responsible for 'N' hormone secretion. However, the more recent work of Venning

et al. (479), demonstrating that methyltestosterone decreases the rate of excretion of urinary corticoids as well as 17-ketosteroids, weakens this argument.

The contrasting patterns of dysfunction of metabolism and of urinary excretion of steroids in Cushing's syndrome and in the adrenogenital syndrome are presented as additional evidence in support of the dual concept. Cushing's syndrome is characterized by a secretion of an excess of 'S' hormone and the adrenogenital syndrome by an excess of 'N' hormone. Albright presumes that each of these pathological states is the result of an exaggeration of one of the dual functions of the normal gland.

The X zone of the adrenal of the mouse has been suspected of having an androgenic function (see review by Parkes (333)). Recently, Jones (221) has demonstrated that the zone is under the influence of a pituitary gonadotrophin, most probably LH. However, the experiments of Jones (221) indicate that the X zone does not secrete an androgen, since in hypophysectomized, castrated mice whose X zone is maintained in a histologically normal state with gonadotrophin, the seminal vesicles are atrophied to the same extent as those of untreated hypophysectomized, castrated mice. Large doses of an extract of the anterior pituitary, rich in adrenocorticotrophic and gonadotrophic hormones, produce no change in the prostate or seminal vesicles of the adult castrated rat (128). It appears unlikely that an important physiological role can be assigned to pituitary regulation of androgen secretion by the adrenal cortex of the mouse. Neither is it likely to have an important role in man since a compensatory increase in the secretion of the 'N' hormone, if it does occur, is not of sufficient magnitude to prevent the development of deficiency symptoms following castration of the human male.

III. *Autonomy Concept.* Following hypophysectomy, the glomerulosa zone of the adrenal cortex thickens, whereas the fasciculata shrinks markedly and the reticularis disappears almost entirely (77, 154); phosphatase disappears from the zona fasciculata but persists in the zona glomerulosa (84); the staining reactions for 'ketosteroids' persist in the glomerulosa but fade in the fasciculata (154). These morphological and cytochemical observations together with the fact that the hypophysectomized rat, in contrast to the adrenalectomized animal, can survive without salt therapy has prompted Swann (451) and Greep and Deane (77, 153, 154) to assign to the glomerulosa zone the specific function of autonomously elaborating hormone regulating electrolyte balance; ACTH is limited in its action to the fasciculata from which is secreted hormone influencing carbohydrate metabolism. The concept implies that the character of a metabolic disturbance can determine the nature of the secretion of the adrenal cortex.

Greep and Deane and their associates have presented experimental evidence in support of the autonomy concept. Desoxycorticosterone acetate (DCA) depletes the lipid of the cells of the glomerulosa of the hypophysectomized rat, a fact interpreted to mean that the zone atrophies following administration of a steroid closely related in function to that which the zone normally secretes (153). The cells of the glomerulosa are claimed to become more than normally active when the animal is fed a diet low in sodium, as shown by lipid depletion and enlargement of the cells. On the other hand, a diet low in potassium produces histological changes which have

been interpreted to signify atrophy. The intraperitoneal injection of potassium induces lipid depletion and broadening of the glomerulosa within a few hours after its injection (79). Finally, according to Ingle *et al.* (205, 213) and Bergner and Deane (28), ACTH has little or no effect upon electrolyte metabolism in the rat.

The survival of the hypophysectomized organism without salt therapy deserves further comment. The concept of autonomous regulation of the secretion of DCA-like steroids fails to explain why panhypopituitarism, in animals or patients, very definitely impairs the ability to adjust to situations of electrolyte stress. Hypophysectomized rats are hypersensitive to the intraperitoneal injection of glucose, a measure which depletes plasma sodium (222, 375). Patients with panhypopituitarism are unable to cope with an excess of potassium or a deficiency of sodium (445), and sweat excretion of sodium in this disease is greater than normal, although less than that which occurs in patients with Addison's disease (58).

Cheng and Sayers (51) have confirmed the observation that DCA depletes the lipid of the glomerulosa zone of the adrenal of the hypophysectomized rat. However, in a number of hypophysectomized rats, thyroxine and estrogen had the same effect. Jones (220) has demonstrated that testosterone depletes the lipid of the glomerulosa of the hypophysectomized mouse. The specificity of action of DCA on the glomerulosa is placed in doubt by these observations; the lipid depletion may be a manifestation of a direct cytotoxic action. It is of interest in this connection that a low-potassium diet as well as a low-sodium diet depletes the lipid of the glomerulosa zone (316).

The fact that DCA induces atrophy of the fasciculata (153) is difficult to reconcile with the autonomy concept. Furthermore, the signs of hypertrophy of the glomerulosa zone which accompany the feeding of a sodium-deficient diet cannot be prevented by the administration of DCA (79). Recently Greep and Deane (155) have shown that the glomerulosa zone can regenerate cortical tissue which is capable of maintaining blood sugar at a normal level during fasting, evidence which suggests that the glomerulosa zone is potentially capable of secreting 11,17-oxysteroids. Finally, highly purified ACTH influences the concentration of sudanophilic material in the cells of the glomerulosa zone of the adrenal cortex of the mouse (221).

The role of the zones of the adrenal cortex remains an interesting enigma. The reviewer considers it premature to assign distinct secretory functions to the zones when the problem of the nature of the secretion of the adrenal cortex is not yet settled. It becomes increasingly difficult with the accumulation of new experimental data in the field to accept desoxycorticosterone as a natural product of the gland. The possibility should be considered that the zones represent different phases of vegetative mitotic activity, growth, maturation and aging (508).

IV. *Unitarian Concept.* The unitarian point of view regards the secretion of the adrenal cortex as relatively fixed in composition but varying in rate. Furthermore, the possibility is entertained, admittedly without conclusive evidence, that a single steroid can account for all the metabolic activities of the gland.

At first thought it is tempting to divide the various metabolic actions of the adrenal cortex among 11,17-oxysteroids, DCA-like steroids and androgens. The conclusion has been reached by many that at least two major types of steroids,

11,17-oxysteroids and DCA-like steroids, must be secreted by the gland in order to explain the diabetogenic as well as the sodium-retaining properties of ACTH in man. However, it would be unwise to be dogmatic on this point at the present time, for it has been shown by Forsham *et al.* (123) that cortisone can induce sodium retention in patients with Addison's disease.

The opinion prevails among clinical investigators that the normal adrenal cortex secretes androgen. However, the experimental and clinical data presented in support of this point of view may be explained just as reasonably on the basis of conversion of cortical hormone to androgens by the liver or other tissues. Administration of cortisone (123) and of ACE (91) elevates the 17-ketosteroid excretion of patients with Addison's disease, and the injection of large doses of cortisone increases 17-ketosteroid excretion (346) and produces mild hirsutism (441) in female subjects with intact adrenals. Furthermore, as has been pointed out in a previous section, the fact that 17-ketosteroids with an oxygen on C-11 have been isolated from urine (262, 263, 282) suggests that androgens may arise from the degradation of cortical hormone.

The relatively high androgen titers in the body fluids in Cushing's syndrome, as manifested by acne and hirsutism, may be explained by the metabolic degradation of 11,17-oxysteroids to adromimetic steroids. On the other hand, the exceedingly high titers of androgens which characterize the adrenogenital syndrome are most likely a result of an aberrant secretory activity of the adrenal cortex itself or of a neoplasm of the gland. The common site of origin of the gonads and adrenals in the embryo may have some bearing on the occasional occurrence of androgen-secreting tissue in the adrenals, an anomaly of nature which has no necessary relation to the normal physiology of the adrenal cortex. Another possible explanation of the adrenogenital syndrome should be kept in mind. The syndrome could be a consequence of an abnormality in the mechanisms concerned with the degradation of the cortical hormone in the liver or other tissues such that the conversion of cortical hormone to androgens proceeds at an abnormally high rate. If this explanation is true, the phenomenon is an example of an endocrinopathy arising as a result of a defect in the utilization of hormone by effector cells.

The nature of the secretion of the adrenal cortex remains in doubt. When the steroid chemists turn their attention from the analysis of dead tissue to the analysis of adrenal effluent blood they will undoubtedly help to elucidate the problem. A beginning has been made in this direction. Nelson *et al.* (313) find only 17-hydroxycorticosterone and possibly a small amount of cortisone in adrenal vein blood of the dog. It is perhaps too much to hope that all the complexities regarding the composition of the adrenocortical secretion have been products of man's imaginations and that a single or at most a few steroids mediate the functions of the gland!

Regulation of Rate of Secretion of Adrenocortical Hormone

I. *Pituitary Regulation of Secretory Activity of the Adrenal Cortex.* It has been firmly established that discharge of ACTH from the adenohypophysis is an essential link in the series of events, initiated by stress, which activate the adrenal cortex. Following hypophysectomy, the adrenal cortex atrophies (77). Stress, which normally

increases the size and decreases the concentrations of sudanophilic substance, cholesterol and ascorbic acid of the adrenal cortex, fails to influence the gland in the absence of the pituitary (190, 400). The adrenal cortex of the hypophysectomized animal is inert to stress although the gland undoubtedly continues to secrete cortical hormone, albeit in minute quantities and at a steady rate. The important role of the adenohypophysis in the response of the organism to stress is indicated by the fact that the hypophysectomized animal, like the adrenalectomized animal, is extremely sensitive to a variety of non-specific stresses (18, 63, 222, 352, 375, 476) and that ACE increases the resistance of hypophysectomized rats to cold (18, 476). However, it should be pointed out that the school of thought (153, 451) which regards the glomerulosa zone of the adrenal as an autonomous regulator of electrolyte balance objects to such a general conclusion and claims that the hypophysectomized, in contrast to the adrenalectomized animal, can efficiently resist disturbances of electrolyte metabolism.

The trophic factor which stimulates the adrenal cortex has been isolated as a homogeneous protein from sheep (256) and hog (403) pituitary tissue. No substance other than ACTH has been demonstrated to have a direct trophic action on the adrenal cortex. In particular, no gonadotrophin has been shown to stimulate the adrenal cortex in the absence of the pituitary, except in the special case of the mouse where a gonadotrophin acts directly upon the X zone (221). There is no evidence from chemical fractionation studies to support the notion of Selye (425) that the pituitary elaborates a 'glucocorticotrophin,' a 'mineralocorticotrophin,' a 'lipocorticotrophin' and a 'testocorticotrophin.'

Highly purified ACTH will restore the weight and histology of the adrenals of the hypophysectomized rat to a status indistinguishable from normal (396). Sayers and Sayers (396) claim that ACTH can reproduce all the gross, histological and chemical changes which have been observed to occur in the adrenal cortex under a variety of conditions of stress. However, Skelton and co-workers (436) disagree with this point of view and have presented evidence to demonstrate that estrogen will produce alterations in the adrenal cortex which cannot be accounted for by pituitary activation alone. The doses of estrogen employed were high and it is not unreasonable to suppose that toxic doses of estrogens or other steroids might exert a direct action on a gland engaged in the synthesis of related substances.

In man, purified ACTH induces all the metabolic changes which have been ascribed to the adrenal cortex. ACTH administration results in metabolic changes characteristic of those produced by the 11,17-oxysteroids (diabetes mellitus-like changes in carbohydrate metabolism (40, 60, 124, 287, 394), sodiumphoresis (124, 394), lymphocytopenia and eosinopenia (124, 180, 394)), by DCA (sodium retention (60, 124, 287, 364, 394)), and by androgens (acne and hirsutism (60, 124, 284)). The trophin increases the urinary excretion not only of corticoids, but also of 17-ketosteroids (40, 284, 394, 469, 479). It would appear that a single trophin, the currently isolated and characterized ACTH, can account for the numerous metabolic actions which have been associated with adrenocortical activity.

A few minutes after the administration of ACTH there occurs a measurable reduction in the concentration of ascorbic acid in the adrenal. The degree of deple-

tion is proportional to the quantity of hormone administered (404). Likewise, application of stress is followed within a few minutes by a depletion of adrenal ascorbic acid, a result of discharge of ACTH from the pituitary. The degree of depletion of adrenal ascorbic acid is proportional to the intensity of the stress (395, 397). Other indices of ACTH activity—depletion of sudanophilic substance, decrease in concentration of cholesterol and increase in glandular weight—also occur in response to stress. The conclusion is almost inescapable that the titer of ACTH in the blood must be elevated in response to stress, although present bioassay technics are not sensitive enough to establish positively the presence of ACTH in the blood of either stressed or non-stressed animals (51). One hour following the subjection of an animal to a severe stress, the normally large store of ACTH in the adenohypophysis is reduced approximately one half, a phenomenon which is undoubtedly the result of discharge of hormone from the gland (298).

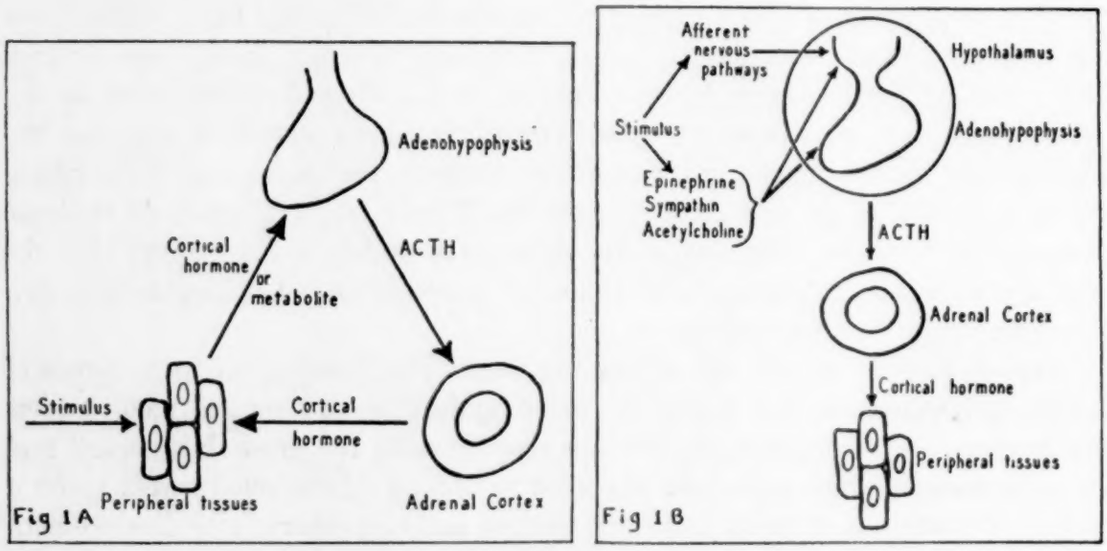

Fig. 1*A*. SELF-REGULATORY system of the peripheral-humoral concept
Fig. 1*B*. CENTRALLY-DRIVEN system of the central-neural concept

II. *Regulation of Pituitary Adrenocorticotrophic Activity.* The question arises as to the nature of the regulatory mechanism which determines the rate of discharge of ACTH. The subject will be discussed under the following headings: peripheral-humoral, central-neural and central-neurohumoral mechanisms. The role of the sympatho-adrenal system in the regulation of pituitary adrenocorticotrophic activity will be given special consideration. The classification is not entirely satisfactory but appears to be as adequate as present experimental data will permit. The proponents of a peripheral mechanism stress the rate of utilization of cortical hormone by the tissue cells of the organism as the determining factor; rate of utilization influences the titer of hormone in venous blood which in turn regulates the rate of release of ACTH from the adenohypophysis. The proponents of the central mechanisms emphasize the role of the central nervous system in initiating the discharge of ACTH. The essential features of the two concepts are diagrammatically illustrated in figure 1 as a self-regulatory system on the one hand, and a centrally-driven system on the other.

1) PERIPHERAL-HUMORAL MECHANISM. A considerable body of indirect evidence

can be marshaled in support of the concept that the titer of cortical hormone in the body fluids regulates the rate of discharge of ACTH from the adenohypophysis. Adrenal atrophy follows the chronic administration of ACE (201, 202) or DCA (82, 390, 414, 485, 486). DCA inhibits the hypertrophy of the adrenals which normally follows the application of a variety of non-specific damaging agents (424), exercise (26, 424), thyroxine (182), estrogen (4) or electroshock-induced convulsions (511). The adrenal hypertrophy which follows thyroxine administration is inhibited by treatment with ACE (494). In man, withdrawal of DCA is followed by metabolic changes characteristic of Addison's disease (516). These chronic experiments may be interpreted to mean that cortical steroid administration inhibits pituitary adrenocorticotrophic activity under optimal and stressful conditions.

Ingle (190) was able to show that the hypertrophy of the adrenal cortex of the rat, which normally occurs after 12 hours of forced exercise, does not occur when animals are treated with ACE throughout the period of exercise. Sayers and Sayers (395) have demonstrated that the drop in adrenal ascorbic acid, which takes place one hour after the application of cold or of heat, or after the injection of typhoid toxin, epinephrine or histamine, can be prevented by pretreatment of the animal with ACE or crystalline cortical steroids. ACE has been shown by Long and coworkers to inhibit the depletion of adrenal ascorbic acid that normally occurs after exposure to cold or after unilateral adrenalectomy (268) or administration of epinephrine (267). ACE administered just prior to exposure to x-irradiation will prevent the early decrease in the concentration of adrenal cholesterol which otherwise follows such exposure (452). The lymphocytopenic action of epinephrine can be inhibited by ACE (137). These numerous confirmatory observations, in which a number of different stresses and indices of adrenocortical activity are employed, strongly suggest that the great variety of non-specific stresses stimulates the adrenal cortex to activity by increasing the requirement of the organism for cortical hormone. Administration of cortical steroid obviates the necessity for the adrenal cortex to increase its secretory activity to meet the increased demands for hormone induced by stress.

Since the trophic action of ACTH on the adrenals of the hypophysectomized animal, as measured by increase in gland weight (201, 202) and ascorbic acid depletion (395), is not influenced by administration of ACE it is reasonable to assume that cortical hormone acts to inhibit release of ACTH from the pituitary rather than to interfere with the action of the trophin on the adrenal cortex itself.

Discharge of ACTH in response to moderate stress may be completely or partially blocked depending upon the dose of cortical steroid administered. Furthermore, with increasing intensity of stress, the amount of cortical steroid required to suppress pituitary adrenocorticotrophic activity becomes correspondingly greater (395, 396). These quantitative relationships may be adequately explained if it is assumed that the rate of discharge of ACTH from the adenohypophysis fluctuates in accordance with the varying requirements of the organism for cortical hormone, i.e., the pituitary-adrenocortical system maintains the tissues in a state of well-being in regard to adrenocortical hormone ('eucorticism') under optimal and stressful conditions. The following working hypothesis seems to fit the available facts.

Under optimal conditions of the environment the pituitary-adrenocortical sys-

tem is in a relatively quiescent state. The peripheral tissue cells under these conditions require relatively small amounts of cortical hormone and the concentration of the latter in venous blood is maintained at a level which holds the anterior pituitary in check. Minimum amounts of ACTH are elaborated by the anterior pituitary and minimum amounts of cortical hormone are secreted by the adrenal cortex.

In contrast, the pituitary-adrenocortical system is very active under conditions of stress. The rate of 'utilization' of cortical hormone by the tissue cells is increased and hence the quantity in venous blood is temporarily reduced. The adenohypophysis responds by discharging ACTH at an accelerated rate. Increased secretory activity of the adrenal cortex occurs and thus the increased needs of the peripheral tissue cells for cortical hormone are met. The titer of cortical hormone in venous blood is presumed to remain fixed within a relatively small range. The exact degree of sensitivity of the pituitary-adrenocortical system is unknown but a certain degree of fluctuation from slightly elevated to slightly reduced titers of cortical hormone may be expected to occur particularly during periods when the requirements of the tissue cells are undergoing rapid change. Increased activity of the pituitary-adrenocortical system continues until the stress is removed or until adaptation occurs, at which time the requirement of the peripheral tissue cells for cortical hormone is diminished.

The peripheral-humoral concept of regulatory control considers the activity of the adrenal cortex to be dictated by the requirements of the tissue cells of the organism for cortical hormone. The concept draws a sharp distinction between the activity of the adrenal cortex and the status of the tissue cells in regard to cortical hormone; actually there is no necessary relation between the two. For example, it is possible for a hyperactive gland to be associated with hypo-, eu-, or hypercorticism. A normal pituitary-adrenocortical system appears to be able to maintain eucorticism under most circumstances, although the possibility exists that in certain types of severe stress there is an actual deficiency of cortical hormone, as will be discussed at greater length in another section.

Support for the peripheral-humoral concept comes from the work of Taylor *et al.* (457) who have been able to demonstrate ACTH in the blood of untreated patients with adrenocortical insufficiency; the blood of normal subjects did not contain detectable quantities of hormone.

The cortical hormone may act directly on the adenohypophysis or indirectly through some metabolite which varies in concentration as a result of an excess or a deficiency of the hormone. The multitude and complexity of changes which can occur in the blood bathing the pituitary gland precludes a ready answer to the problem. Sayers and Sayers (395) have suggested as a working hypothesis that the action of the cortical hormone on the pituitary is a direct one. The suggestion was first made on the basis of the fact that inhibition of pituitary adrenocorticotrophic activity was induced by both cortisone and desoxycorticosterone despite the fact that they appear to be qualitatively distinct in their actions on carbohydrate metabolism.[4]

[4] The cortical steroids with an oxygen on C-11 induce glycogen deposition; in contrast, even large amounts of DCA have no activity in this test (149, 328, 330). Wick *et al.* (505) reported that DCA has no influence upon the action of insulin in the diabetic rat and does not exacerbate the

From such a working hypothesis a corollary is at once obvious. Administration of DCA, by suppressing pituitary adrenocorticotrophic activity and thereby the secretory activity of the adrenal cortex, should result in a steroid hormone imbalance characterized by an excess of DCA (administered steroid) and a deficiency of endogenously secreted hormone which influences carbohydrate metabolism. Indeed, Cheng and Sayers (50) have demonstrated that chronic DCA administration induces a state of insulin hypersensitivity and Woodbury *et al.* (511) have shown that the elevation in plasma sodium and the reduction in excitability of the central nervous system which accompany DCA treatment may be restored to normal by the simultaneous administration of ACTH or whole extract of the adrenal cortex.

A number of apparently anomalous reports on the effect of DCA on carbohydrate metabolism may be explained on the basis of this working hypothesis. Chronic treatment with DCA has been shown to impair the ability of the rat to mobilize glucose when the animal is subjected to a variety of non-specific types of stress (424). DCA increases the glucose tolerance of dogs and rabbits made diabetic with alloxan (239), of normal subjects (237), and of diabetic patients (235, 238). The steroid has been shown to reduce the insulin requirement of diabetic patients (510) and to increase the arterio-venous difference in concentration of glucose in normal subjects given glucose plus insulin (236). DCA inhibits the hyperglycemic actions of epinephrine (234) and posterior pituitary hormone (233). McGavack *et al.* (290) found that DCA influences the response of normal human subjects to oral glucose; the administration of DCA caused "an early higher rise and a marked depression approaching hypoglycemic levels in the fourth and fifth hours."

Also of interest in this connection are the observations of Ingle and Thorn (216) that DCA in doses of 1 to 5 mg. per day reduces the urinary nitrogen excretion

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diabetes of animals from whom insulin was withdrawn. On the other hand, two groups of investigators have reported that DCA has a 'diabetogenic' action. Ingle and Thorn (216) found that administration of doses of 1, 2 or 5 mg. per day of cortisone is followed by enhanced glycosuria in partially depancreatized rats, whereas similar doses of DCA have no effect. However, 10 mg. of DCA does produce a definite exacerbation of the glycosuria. More recently, Ingle (197) has demonstrated that the glycosuria of depancreatized rats is reduced by the daily administration of 2 mg. of DCA but exacerbated by 10 mg. Wells and Kendall (497) demonstrated that DCA, although not quite as effective as cortisone, very definitely increased the glycosuria of the phlorhizinized adrenalectomized rat. The experiments support the view that large doses of DCA have a diabetogenic effect, i.e., a metabolic action similar to that of the cortical steroids with an oxygen on C-11. However, it is important to note that these two groups of investigators have employed glycosuria as an index of 'diabetogenic' action. Large doses of DCA may produce the 'diabetogenic' effect through a renal mechanism, i.e., by inhibiting the renal tubular reabsorption of glucose. The possibility takes on added significance in the light of the experiments of Wells and Kendall (497) who noted that cortisone markedly increases the glycosuria of phlorhizinized adrenalectomized rats and at the same time prevents the collapse, convulsions and death which frequently occur in untreated controls. On the other hand, DCA produces a definite although less marked glycosuria, but is entirely ineffective in preventing coma, convulsions and death. This may be interpreted to mean that DCA does not benefit the adrenalectomized animals by elevating the blood sugar but rather acts synergistically with phlorhizin to inhibit reabsorption of glucose. Of interest in this connection is the demonstration of Lambert *et al.* (243) that DCA reduces the renal threshold for glucose in man.

Cheng and Sayers (51) have been unable to confirm the results of Harrison and Harrison (165) that DCA in large doses maintains the blood sugar of the fasting adrenalectomized rat. Indeed, Cheng and Sayers found that DCA impairs the ability of intact rats to sustain normal blood sugar levels during a prolonged fast.

of partially depancreatized rats with intact adrenals, although a dose of 10 mg. per day increases the rate of nitrogen excretion. Conn *et al.* (59) and Oberdisse and Werner (324) have reported reduction of urinary nitrogen in men receiving DCA.

The observations just cited suggest that DCA induces a metabolic derangement characteristic of a deficiency of the 11,17-oxysteroids. Part of the action is undoubtedly a result of suppression of pituitary adrenocorticotrophic activity; however, the possibility that DCA and 11,17-oxysteroids compete for loci in effector cells must also be considered.

Certain observations which concern the problem of regulation of pituitary adrenocorticotrophic activity require special consideration. Although not necessarily incompatible with the humoral concept, they point up the necessity for further investigation in this field. Pretreatment with relatively large doses of cortical steroid partially but not completely blocks the reduction in adrenal ascorbic acid which normally occurs when rats are given large doses of histamine (395). Administration of ACE prevents the decrease in concentration of adrenal cholesterol which occurs in the first few hours after x-irradiation but fails to prevent the late adrenal changes (452). Depletion of cholesterol in the adrenals of rats infected with *B. tularense* is uninfluenced by large doses of ACE given at frequent intervals (357). However, administration of ACE prevents the adrenal hypertrophy which normally follows exposure of rats to low atmospheric pressure for two to three days (248). Adrenal hypertrophy does not occur in the fasted rat given DCA (71), but neither DCA (71) nor ACE (69, 70) affects the hypertrophy of fasting in the guinea pig. The following explanations of these experimental facts may be considered. First, it is entirely possible that in the experiments where complete inhibition of pituitary adrenocorticotrophic activity is not obtained, insufficient quantities of cortical steroids are employed; rate of 'utilization' of cortical hormone appears to be exceedingly great during severe stress. Second, it may well be that a peripheral-humoral mechanism is the only mechanism at work in mild to moderate degrees of stress, whereas in more severe degrees of stress other mechanisms, e.g., central-neutral, play a complementary role to accelerate the rate of discharge of ACTH. Third, in severe stress with accompanying cardiovascular collapse, anoxia or accumulated toxins may act directly upon the cells of the adenohypophysis to increase their permeability to ACTH.

Moya and Selye (307) have presented evidence which they interpret to be incompatible with the peripheral-humoral concept. They found that DCA treatment would not prevent the reduction in adrenal ascorbic acid which normally follows exposure of rats to cold. However, examination of their data shows that a suprisingly small reduction in adrenal ascorbic acid occurred in untreated animals exposed to cold. Furthermore, the doses of DCA employed were enormous and sufficient to 'anesthetize' the rats. The physiological significance of such an experiment remains in doubt. It would not be at all surprising to find that physiological levels of the cortical steroids have an inhibitory influence upon the pituitary, whereas toxicological doses, which produce general collapse of the organism, exert a toxic action on the adenohypophysis with release of stored ACTH.

Corcoran and Page (61) have found that adrenal vein blood has a much higher

concentration of chemocorticoids (formaldehyde-generating lipids) than does peripheral venous blood. However, administration of epinephrine in dogs induces an elevation in the titer of chemocorticoids in both adrenal and peripheral venous plasma; in stressed animals, as in normal animals, there is a much higher concentration of chemocorticoids in adrenal vein blood than in peripheral vein blood. Since the method of analysis of chemocorticoids does not distinguish biologically active from inactive steroids, it is entirely possible that the elevated titer of corticoids in the venous blood after stress is due to increased quantities of degradation products of the cortical hormone. The solution of the problem requires further refinement of methods of analysis of cortical hormone in blood.

The peripheral-humoral concept implies that a normal pituitary-adrenocortical system does not allow an organism to be subjected to a plethora of cortical hormone, such as is suggested by the 'diseases of adaptation' thesis of Selye (418). The problem appears to resolve itself into the question of how narrow a range of titers of cortical hormone exists in the peripheral venous blood of animals with a normal pituitary-adrenocortical system under various conditions. Just as a normal pancreas regulates the blood sugar level within a narrow but nevertheless definite range, so the pituitary-adrenocortical system probably maintains eucorticism not at a fixed level but at a narrow range of cortical hormone concentrations in venous blood.

2) CENTRAL-NEURAL AND CENTRAL NEURO-HUMORAL MECHANISM. The rapidity with which ACTH is released in response to stress suggests the possibility that a neural or neuro-humoral factor is involved in acute situations. Cheng *et al.* (52), employing the adrenal ascorbic acid-depletion technic, demonstrated that section of the pituitary stalk in rats does not prevent the discharge of ACTH which occurs within an hour following histamine injection. Furthermore, ACTH is discharged from anterior chamber or splenic transplants of the adenohypophysis in response to acute stress (53). The degree of depletion of adrenal ascorbic acid is not as great in the animal with a transplant as in the normal animal. The reduced response may be due to one or a combination of the following factors: 1) the transplanted tissue is no more than $\frac{1}{20}$ the mass of the normal pituitary and hence stores and/or synthesizes less than normal quantities of hormone; 2) the blood supply to the transplanted tissue is inadequate; 3) the adrenal cortex of the transplant animal, whose metabolic rate is low, may not be as responsive to ACTH as the gland of a normal animal. However, whatever the cause of the decreased response of the transplant animal, it is obvious that the integrity of neural or neuro-vascular links between the hypothalamus and adenohypophysis is not essential for the discharge of ACTH. The experimental evidence cited does not rule out the possibility that neural factors may modify the rate of discharge of ACTH from the pituitary. The effect of discrete lesions in the hypothalamic area on the adrenocorticotrophic activity of the adenohypophysis may help to elucidate this problem. In a preliminary report Hume (188) claims that, whereas disruption of neural and vascular connections between the hypothalamus and the pituitary in dogs does not interfere with the function of the pituitary-adrenocortical system as measured by the eosinophil test, certain specific lesions of the hypothalamus (anatomical location not given) abolish the usual eosinopenic response to stress. Hume suggests that the hypothalamus releases a neuro-humor when acti-

vated by stress; the neuro-humor acts on the adenohypophysis to bring about discharge of ACTH (fig. 1*b*).

Section of the infundibulum has no influence upon the release of ACTH induced by chronic exposure to cold (38, 477).

3) Role of Sympatho-Adrenal System. The close anatomical approximation of the adrenal medulla and cortex together with the fact that they both play important roles in homeostasis naturally leads to some speculation regarding a possible integrative functional relation between the sympatho-adrenal and pituitary-adrenocortical systems.

Vogt (488), from studies on the biocorticoid (cold-protection test) content of adrenal vein blood reached the conclusion that epinephrine has a direct stimulatory influence upon the adrenal cortex. However, her experiments do not rule out the possibility that epinephrine acts via the adenohypophysis to bring about a discharge of ACTH. Necessary but not sufficient evidence for the direct action thesis is the observation that epinephrine induces a lymphocytopenia in the hypophysectomized rat (189) and an eosinopenia in the hypophysectomized mouse (438). On the other hand, ACTH, but not epinephrine, stimulates the production of chemocorticoids by the isolated perfused adrenal gland (172).

Long (267, 268) has pointed out that both the sympatho-adrenal and pituitary-adrenocortical systems are stimulated to increased activity by a great variety of stressful conditions, and he is inclined to the view that epinephrine ". . . is not in the usual sense 'a non-specific agent.'" "The stimulation of the elements of the autonomic nervous system with concomitant release of epinephrine that occurs under a variety of conditions appears to be a major factor in the activation of the adrenotrophic secretion from the anterior lobe. The manner by which epinephrine produces this activation is now known. A further regulatory factor is the blood level of cortical hormones."

Our current knowledge includes the following possible mechanisms for the action of epinephrine in inducing discharge of ACTH from the adenohypophysis: *1*) epinephrine may act centrally on effector cells in the adenohypophysis or through the hypothalamus which in turn would activate the adenohypophysis; *2*) epinephrine may act like other non-specific agents and stresses to increase tissue utilization of cortical hormone with a consequent lowering of venous titer of the hormone; *3*) epinephrine may be the denominator common to all types of stress and the specific agent which promotes utilization of cortical hormone by the tissues; and *4*) epinephrine may act by any one of various possible combinations of these three.

Sayers and Sayers (396) have shown, and Long (267) has confirmed, that the reduction in adrenal ascorbic acid produced by epinephrine can be prevented by administration of cortical steroid. The lymphocytopenic action of epinephrine is prevented by cortical steroid treatment (137). These observations strongly suggest that epinephrine acts like other non-specific stresses and does not have a direct action upon the pituitary.

Neither Dibenamine, an agent which blocks the excitatory effects of both sympathin and epinephrine (320), nor tetraethylammonium bromide, a substance which produces autonomic ganglionic blockade (1), influences the reduction in adrenal

ascorbic acid which takes place in the rat in response to acute stress (458). The lymphocytopenia of tourniquet shock was of greater magnitude in rats treated with Dibenamine than in animals not treated with the adrenergic blocking agent (506). These observations do not lend support to a direct action of epinephrine on the adenohypophysis or associated structures; however, negative experiments of this nature are not conclusive since it is possible that the action of epinephrine on pituitary effector cells is not influenced by the autonomic blocking agents employed.

The completely sympathectomized animal is an important experimental tool which has not yet been fully exploited in the elucidation of the role of the sympatho-adrenal system in the regulation of pituitary-adrenocortical activity. It would be of great interest to know the comparative responses of the adrenal cortices of intact and sympathectomized animals to both acute and chronic types of stress. Cannon (47) has demonstrated that the sympathectomized cat is hypersensitive to various environmental exigencies. However, the degree of hypersensitivity does not approach that of the adrenalectomized animal. The completely sympathectomized dog is hypersensitive to insulin, but resists heat, cold and anoxia almost as well as do intact control animals (289). Furthermore, the sympathectomized dog is capable of maintaining normal blood sugar levels during exercise (39). Homeostasis is not significantly threatened by total sympathectomy in man (369). These studies suggest that acceleration of adrenocortical activity in response to acute stress is not dependent upon the activity of the sympatho-adrenal system; neither does it appear that epinephrine is essential for the action of adrenocortical hormone on effector cells, for the addition of epinephrine to an infusion of ACE did not improve the muscular work performance of adrenalectomized rats over that of similar animals given ACE alone (209). Whereas the sympatho-adrenal system is essential neither for the functional activity of the pituitary-adrenocortical system nor for the response of effector cells to cortical hormone, the integrity of the pituitary-adrenocortical system appears to have a considerable influence upon the response of certain effector cells to epinephrine. For example, the pressor response to sympathetic stimulation is diminished by adrenalectomy and restored to normal by cortical steroid replacement therapy (409).

Gellhorn and Frank (138) claim that hemorrhage and sub-convulsive electroshock cause a lymphopenia in normal but not in adrenodemedullated rats. It is recognized that epinephrine discharge contributes to the increase in the adrenocorticotrophic activity of the adenohypophysis in numerous acute stresses. However, the common association of epinephrine discharge and increased secretory activity of the adrenal cortex does not help answer the important question as to whether the action of epinephrine is a necessary and essential link in the series of events which lead to increased secretion of cortical hormone during stress or whether epinephrine acts like other non-specific stresses to increase the needs of tissues for cortical hormone.

Although the sympatho-adrenal system responds to a wide variety of stresses, the role of this nervous factor is neither as ubiquitous nor as vital in homeostatic adjustment as is the pituitary-adrenocortical system. The sympatho-adrenal system actively drives organs and organ systems to increased functional activity in emergencies, whereas the pituitary-adrenocortical system plays a passive role, making

cortical hormone available in quantities appropriate for the varying needs of the organism. In other words, the sympatho-adrenal system initiates, whereas the pituitary-adrenocortical system supports cellular activities.

The nature of the regulatory mechanism or mechanisms which are concerned with the discharge of ACTH has an important bearing on the treatment of rheumatoid arthritis and other collagen diseases of man. The beneficial effects of cortisone or ACTH appear to be associated with the development of hypercorticism rather than replacement of a hormone deficiency, i.e., the action is pharmacological rather than physiological. The possibility has been entertained that epinephrine, a substance more readily available than ACTH or cortisone, might be of therapeutic benefit by stimulating the discharge of ACTH from the pituitary. Epinephrine may be expected to be of therapeutic benefit if it acts centrally to bring about discharge of ACTH without regard to the rate of utilization of cortical hormone by the tissues or to the titer of hormone in venous blood. On the other hand, if epinephrine acts by increasing the rate of utilization of cortical hormone by the peripheral tissues, then it would be expected to be of slight benefit at most, for eucorticism will prevail after its administration despite hyperactivity of the adrenal cortex, i.e., the tissues will not be flooded with an excess of cortical hormone but rather they will receive quantities of hormone appropriate to the increased needs induced by epinephrine. The elevation in the titer of cortical hormone in arterial blood, necessary to meet the increased requirements, may be expected to have some influence upon the diseased tissues. Thorn *et al.* (465) found that epinephrine resulted in a slight but definite improvement in rheumatoid arthritis, although the effect was far less than that obtained with ACTH. Thorn and associates state that, "In contrast to the uninhibited stimulation of adrenal cortical hormones that follows the repeated administration of ACTH, epinephrine administration has the disadvantage of losing much of its effectiveness as an adrenal cortical stimulator since endogenous ACTH production is inhibited by a rising titer of adrenal cortical steroids."

The titer of cortical hormones in the body fluids appears to play a major role in the regulation of pituitary adrenocorticotrophic activity. The exact nature of the process by which the changing levels of cortical hormone in the blood influence the rate of discharge of ACTH from the adenohypophysis is unknown; it is here that the peripheral-humoral concept is particularly vague. The concept emphasizes the determining role of the peripheral tissues in regulating pituitary adrenocorticotrophic activity by dictating the rate of 'utilization' of cortical hormone. On the other hand, it minimizes the role of central mechanisms which induce ACTH discharge without regard to tissue needs for cortical hormone. The possibility exists that central-neural mechanisms may exert a modifying influence although the evidence on this point is indeed meager. Whether the liver influences the titer of cortical hormones through degradative inactivation cannot be stated; data (118, 150) are scant and bear only indirectly on this important phase of the problem.

Classification of Responses of the Adrenal Cortex to Stress

It is of some practical as well as theoretical interest to determine the patterns of activity of the adrenal cortex in animals and man in numerous experimental and

clinical conditions. The variety of technics which are available to make such studies have been described and evaluated in an early section of this paper. In experimental animals, the adrenals may be removed and analyzed in regard to size, histology and chemistry; mass of lymphoid tissue may be employed as an adjunctive index. In man, the number of circulating lymphocytes and eosinophils and the urinary excretion of corticoids are usually employed as indices of adrenocortical activity. It cannot be too strongly emphasized that all these methods are indirect; interpretations gain validity when they are based upon a variety of confirmatory evidence.

The adrenal cortex can undergo rapid and dynamic alterations in functional activity. Chemical changes in the gland may be detected within a few minutes following application of stress or administration of ACTH; recovery of certain constituents to their original concentration may take place within a few hours. It is for this reason that the following factors must be taken into account in an interpretation of indices of adrenocortical function: *a*) the intensity of the stimulation, *b*) the duration of the stimulation, and *c*) the time, during or after stimulation, at which the adrenals are analyzed. As a working scheme of classification, the responses of the adrenal cortex may be considered to fall into the nine general types presented in diagrammatic form in figure 2. Actually there are various grades of intermediate responses among these types. The classification is intended as a guide to interpret functional activity of the adrenal cortex on the basis of changes in size, sudanophilic substance, cholesterol, and ascorbic acid content of the gland. Other measures of adrenocortical activity, such as degree of lymphocytopenia or eosinopenia, rate of urinary corticoid excretion, etc., have also been considered in the development of the scheme. The admittedly oversimplified representation of the alterations which may occur in adrenocortical activity has been undergoing change (396, 399) and is expected to undergo still further change as new experimental facts are made available.

The first row of pituitary-adrenal units in figure 2 represents the pituitary-adrenocortical system as it exists under continuing optimal conditions of the environment. ACTH is released at a very slow rate, the rate of secretion of cortical hormone is minimal and the tissue cells are in a state of eucorticism; each individual cortical cell has an ample store of precursor material.

In the following discussion it will become apparent that there is a great range of activity of the adrenal cortex associated with eucorticism of the tissue cells; the degree of activity of the adrenal cortex has no necessary relation to the state of the tissue cells in regard to cortical hormone.

Type I. Sudden Temporary Period of Stress. In this category, the stimulus acts for a short period (few hours); there is a sudden, temporary increased demand for cortical hormone resulting in an equally sudden and temporary increase in pituitary adrenocorticotrophic activity. The cells of the adrenal cortex secrete cortical hormone at a rate sufficient to maintain the tissue cells in a state of eucorticism. The sudanophilic substance, cholesterol, and ascorbic acid undergo rapid depletion followed by a return of these substances to normal concentration as the animal recovers. There is little or no increase in the size of the adrenal. Typical examples of this type of adrenal response are provided by an acute nonfatal hemorrhage (400), a short bout of muscular exercise (11, 111, 230), a single dose of very short-acting drugs such as

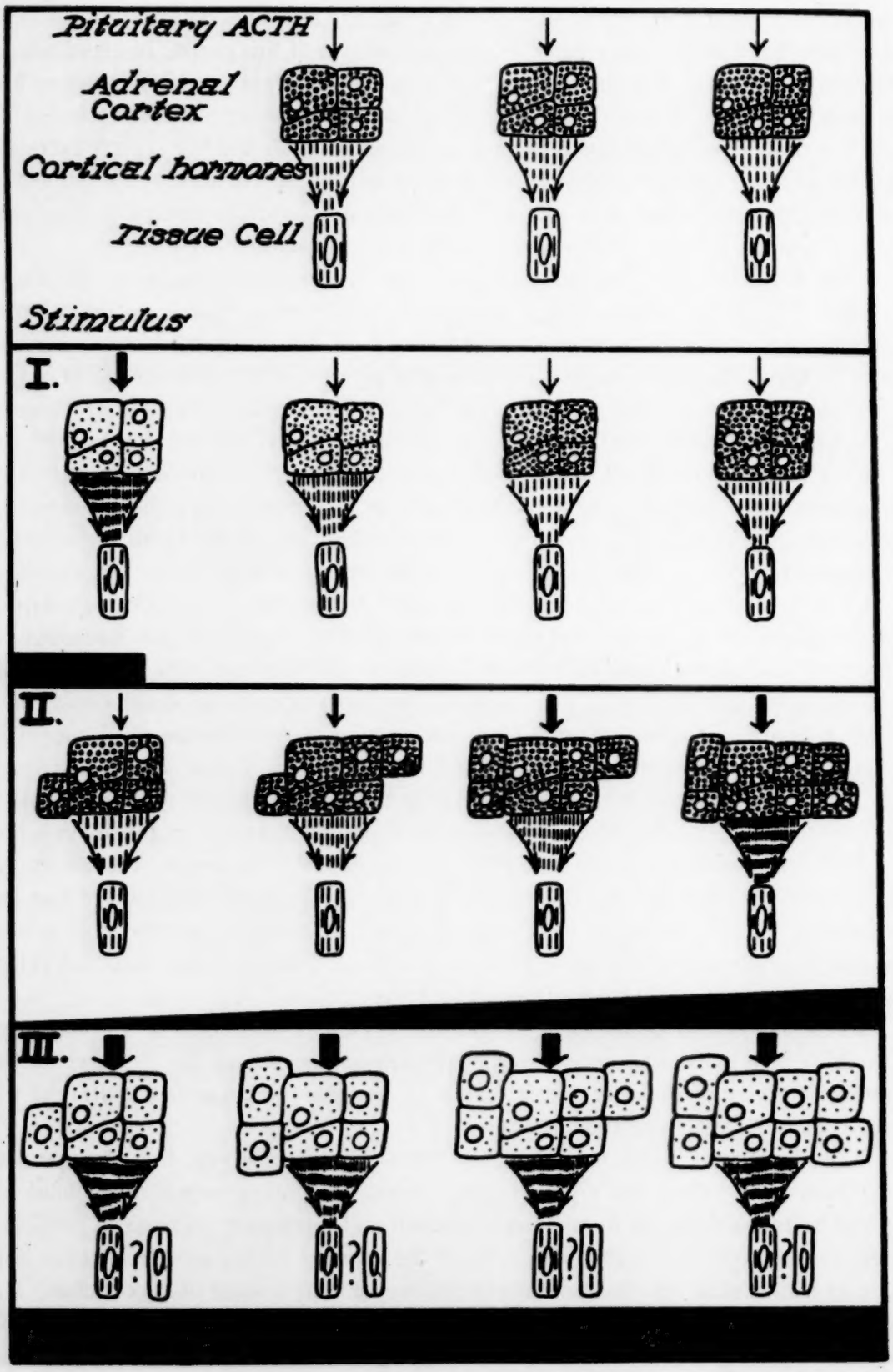
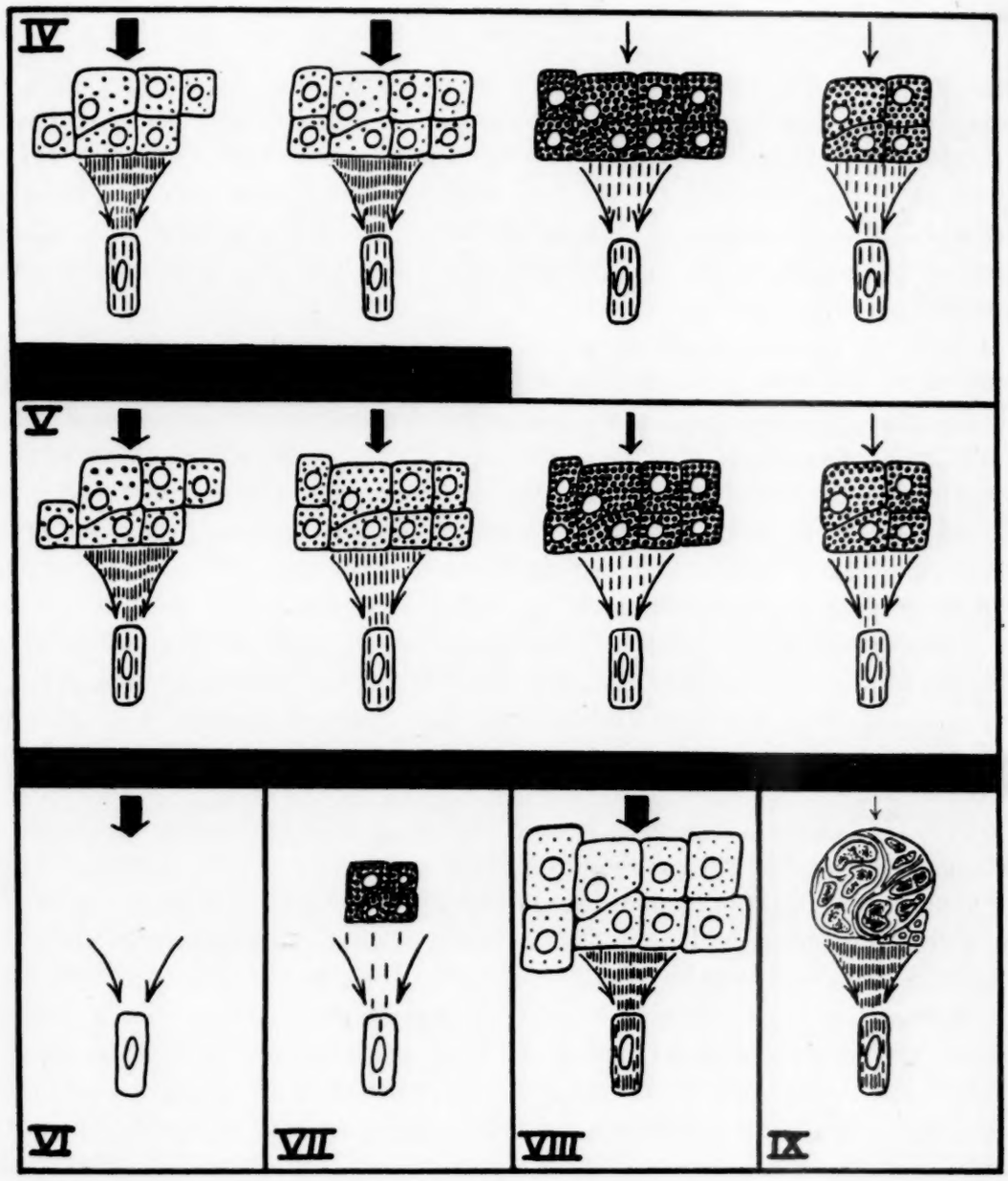

Pituitary ACTH
Adrenal Cortex
Cortical hormones
Tissue Cell
Stimulus
I.
II.
III.
?
?
?
?

FIG. 2. PART 1.



PART 2.

Fig. 2. WIDTH OF UPPER ARROWS is proportional to rate of discharge of ACTH from pituitary. Black granules in cells of adrenal cortex represent stored cholesterol esters, probably the precursor material of cortical hormone; quantity of cholesterol ester is measured by intensity of Sudan stain or by chemical analysis. The gland may undergo hypertrophy or hyperplasia, as illustrated by increase in size of individual cells or by increase in number of cells, respectively. Individual cells are considered as secretory units. Intensity of sudanophilic staining or cholesterol concentration is measure of activity of each unit; depletion of cholesterol is associated with increased activity; accumulation, with hypoactivity. The cortical hormone is shown emerging from the adrenocortical cells as short vertical lines and entering a tissue cell. Number of short vertical lines in cytoplasm of tissue cell represents state of well-being of cells of organism in regard to cortical hormone. Duration and intensity of stimulus is represented by blackened area at bottom of each section. The higher the blackened area the greater the degree of stress. Stimulus is general term which includes stress as well as physiological and commonplace changes of the environment which act to increase activity of pituitary-adrenal system. Stress usually implies a rather severe degree of stimulation; it is to be emphasized that mild stimuli and what are usually considered commonplace happenings of mammalian existence stimulate adrenal cortex to increased secretory activity. Physiological processes such as pregnancy and lactation are accompanied by increase in adrenocortical activity.

epinephrine (269) or histamine (298), low atmospheric pressure (251, 317, 461), and intraperitoneal injection of glucose solution (86). The changes in the adrenal characterized as Type I can be reproduced by the injection of a single dose of ACTH.

Lack of recognition of the dynamic nature of the process involving depletion and mobilization of adrenal cholesterol and ascorbic acid in acute stress explains the apparent discrepancies and varied interpretations of these phenomena which have appeared in the literature.

Type II. Very Gradual Change in Internal or External Environment. In this category, the stimulus gradually increases in intensity over a period of a few weeks or months; there is a very gradual increase in the demand for cortical hormone, which results in an equally gradual increase in pituitary adrenocorticotrophic activity. The concentrations of sudanophilic substance, cholesterol and ascorbic acid of the adrenal remain essentially unchanged. A gradual increase in size of the gland occurs and it is apparent that new secretory units can develop (hyperplasia) at a rate sufficient to meet the increasing demands for cortical hormone without marked depletion of precursor material. The tissue cells are maintained in a state of eucorticism. Typical examples of this type of adrenal response are provided by fasting (277, 304, 310, 325, 326, 450, 475, 504), caloric restriction (36, 110), seasonal changes in temperature (398), pregnancy (12, 367), certain types of high protein diets (460), and mild chronic infection (20).

Type III. Intense Continuous Stress Ending in Death. In this category, the stimulus is intense and continuous; there is a large and continuous demand for cortical hormone resulting in a maximum rate of elaboration of ACTH from the pituitary. The concentrations of sudanophilic substance, cholesterol and ascorbic acid fall rapidly and remain at very low levels until death. The gland develops secretory units at a maximum rate, as evidenced by marked hypertrophy and hyperplasia. The degree of hypertrophy is proportional to the time which elapses between the onset of the stress and death. The state of the tissues in regard to cortical hormone is an unsettled point, hence the reviewer's indecision regarding the representation of the status of the tissue cells (eucorticism or hypocorticism?) in figure 2. A detailed discussion of this subject is undertaken in the section on resistance (see below). Typical examples of Type III adrenal response are provided by lethal doses of toxins (57, 110, 228, 305), infectious diseases leading to a fatal outcome (2, 20, 33, 80, 110, 119, 148, 242, 244, 272, 297, 357, 383, 389, 440, 493, 498), dehydration (318), low atmospheric pressure (251), burns (65, 163, 356), hemorrhagic shock (110, 115, 400) and traumatic shock (87, 308).

Type IV. Recovery From Period of Severe Stress. The pituitary-adrenocortical system is stimulated to greater than normal activity during the period in which a stimulus is acting; the adrenal becomes enlarged and depleted of sudanophilic substance, cholesterol and ascorbic acid. Sudden withdrawal of the stimulus results in the accumulation of precursor material; under this circumstance, the secretory capacity of the gland may be viewed as overexpanded for the needs of the organism under optimal conditions of the environment. The changes in adrenal size and cholesterol concentration characteristic of Type IV response are exemplified by the obser-

vations of Ludewig and Chanutin (273). These workers studied the effect of a single dose of a β-chloro-ethylamine vesicant on adrenal cholesterol in the rat. The initial depletion of the cholesterol content of the gland represents the toxic phase of the response; the return to normal and subsequent increase of concentration above normal represent the recovery phase. The early toxic response and the recovery from thermal injury (274) and from cutaneous application of bis-(β-chlorethyl) sulfide (274) are also accompanied by adrenal changes of this nature. Type IV response can be reproduced by the continuous administration of ACTH for a period of a few days, followed by withdrawal of the hormone (401).

That the phase of accumulation of cholesterol and ascorbic acid in a hypertrophied gland may correspond to the period of increased crossed resistance[5] which follows stress is an intriguing possibility. Quantitative studies relating crossed resistance to adrenal cholesterol and ascorbic acid concentrations are required to elucidate this point.

Type V. Adaptation to Stress. When an animal adapts to a continuously applied noxious stimulus, the adrenal cortex responds in a characteristic manner. Initially there is depletion of adrenal constituents. However, as specific adaptations are made, the stress, although constant and continuously applied, induces less and less change in the internal environment; the requirement for cortical hormone is reduced and the gland returns to a pre-stress state of functional activity; the chemical constituents are restored to normal concentration and may or may not pass through a stage of excess storage. For example, Darrow and Sarason (73) demonstrated lipid depletion of the adrenal cortex during the first 5 days of exposure of rats to anoxia, but lipid material was restored by the seventh to eighth days of exposure. Dalton *et al.* (68) subjected rats to discontinuous exposure to low atmospheric pressure for varying periods up to 12 weeks. Repeated exposure caused adrenal hypertrophy which reached a maximum at six weeks. With continued exposures, the gland gradually returned toward its original size in association with a restoration of normal amounts of lipid, the distribution of which was normal. Levin (251) has shown that exposure for 16 to 22 hours at 0° to 5° C. causes a reduction in the cholesterol concentration of the adrenal, whereas after exposure for 72 hours the concentration of the sterol has been restored to normal in glands which are not significantly different in weight from those of unexposed animals.

Types VI, VII, VIII and IX. These types of adrenal response represent pathological alterations in the pituitary-adrenocortical system; stimuli exert little or no influence. Type VI is a result of partial or complete destruction of adrenocortical cells and is associated with hypocorticism or acorticism of the tissue cells of the organism. Loss of adrenocortical function may be due to dysfunction caused by toxemia, tuberculous destruction, idiopathic atrophy, or hemorrhagic necrosis, of the gland. The maximum rate of discharge of ACTH from the pituitary shown in figure 2 (Type VI) is based on the fact that the blood of patients with untreated Addison's

[5] Crossed resistance to a particular stress is that enhancement of resistance which has been gained by exposure to a different type of stress.

disease but not that of normal subjects contains detectable quantities of ACTH (457). Type VII follows hypophysectomy or destruction of the adenohypophysis. The cells of the cortex become smaller and precursor material is stored in coarse granules. (Deane and Greep (77) describe loss of precursor with prolonged absence of pituitary function.) Even in the complete absence of ACTH, the adrenal cortex continues to secrete a constant minimal quantity of cortical hormone which under optimal conditions may be adequate to maintain the tissue cells in a state approaching eucorticism; but this secretion is inadequate during exposure to stress. Type VIII is associated with pathological hyperactivity of the pituitary. The cells of the adrenal cortex secrete cortical hormone in excess of the tissue needs and thus hypercorticism results. Type VIII can be readily reproduced in man or experimental animals by the administration of ACTH. Taylor *et al.* (457) studied two patients with adrenocortical hyperplasia; the blood of one patient caused a significant decrease in adrenal ascorbic acid in hypophysectomized rats; the blood of the other patient and of normal individuals gave negative results. Additional studies of this nature are awaited with interest. Type IX is a primary dysfunction of the adrenal cortex; a neoplasm, free from regulatory control, secretes cortical hormone in excess of the tissue needs and hypercorticism results. Rogers and Williams (383) find adrenal cholesterol concentration to be low or normal in subjects with hypercorticism.

The system of classification presented here may be of some practical value to the toxicologist in the following three phases of his work. *1*) Alterations in adrenal chemistry may be employed to evaluate the degree of toxicity of a drug at therapeutic dose levels, a measurement which would be a valuable adjunct to the LD_{50}. *2*) Tolerance to the toxic actions of a drug through repeated exposure may be expressed quantitatively by a comparative study of the adrenocortical-stimulating action of equal doses of the drug given to unexposed and exposed animals. *3*) The sequence of adrenal changes may reveal certain characteristics of the mode of action of toxins. Most noxious agents act continuously until the death of the animal. However, other agents have latent periods or biphasic types of action which manifest themselves in the character of the response of the adrenal cortex. For example, Patt and coworkers (338) have recently shown that X-irradiation induces the following changes in the adrenal cortex: an initial short phase of depletion of adrenal cholesterol is followed by a period of recovery lasting a few days; the third and last phase is characterized by marked hypertrophy and depletion of adrenal cholesterol and culminates in the death of the animal.

THE ORGANISM

Increased Rate of Utilization of Cortical Hormone During Stress

Convincing evidence has been presented in previous sections of this review that an increased rate of 'utilization' of cortical hormone is a characteristic and even necessary accompaniment of adequate homeostatic adjustment of the organism to stress. What are the functional alterations induced by stress which increase the need for cortical hormone? Where is the locus of action of the cortical hormone in intracellular processes which confers resistance to stress? The answers to these questions

are not available. It is not even possible at the present time to determine whether the increased rate of 'utilization' of cortical hormone which accompanies stress is *1*) shared by all cells of the organism, *2*) involves only those cells affected by the stress stimulus, or *3*) is participated in by both 'injured' cells and buffer organs attempting to restore homeostasis.

Tepperman *et al.* (459) pointed out that a number of stressful conditions which produce hyperfunction of the adrenal cortex are accompanied by increased catabolism of protein. These same investigators (460) found that a diet high in protein resulted in adrenocortical hypertrophy. They were led to the conclusion that the catabolism of some protein substrate, derived from the animal's own tissues or from dietary protein, increased cortical hormone requirement. Recent experimental work has not substantiated this concept. A number of laboratories (25, 196, 200, 250) have found that not all diets high in protein produce adrenal hypertrophy. Moreover, the fact that the adrenalectomized rat will grow and survive on a diet high in protein (200) is not compatible with the idea that cortical hormone is necessary for the metabolic conversion of protein. The reader is referred to the articles by Ingle (195, 196) in which he has brought together a number of arguments against the 'protein-substrate' concept; he points out that the concept does not distinguish between the cause and the effect of adrenocortical excitation.

Despite the lack of particulars, certain broad aspects of the problem of cortical hormone 'utilization' in stress are worthy of consideration. In general, it would appear that rate of cortical hormone 'utilization' parallels cellular activity and that the hormone is necessary for the mobilization of energy for cellular work. As might be inferred, in adrenocortical insufficiency, cells are incapable of performing functions which require a relatively great expenditure of energy.

Eosinophil counts indicate that the adrenal cortex is hypoactive in myxedema (296), and that thyroidectomy is followed by adrenal atrophy in the rat (314, 365). Thiourea and its congeners induce adrenocortical atrophy in rats (21, 78, 314, 515) and guinea pigs (295). Conversely, the adrenal cortex is hyperactive in hyperthyroidism (203, 296, 494). The facts are compatible with the thesis that cortical hormone 'utilization' parallels cellular activity.

Cellular activities which require a considerable expenditure of energy are seriously impaired in the adrenalectomized animal and in the patient with Addison's disease. Gastric (474), renal (371) and mammary (136) secretory activities and the work capacity of skeletal muscle (193) are markedly diminished.

The increased cellular activity which accompanies the homeostatic adjustments of the organism to stress may be at least partly responsible for the parallel increase in the rate of 'utilization' of cortical hormone. The following experiments are of interest in this connection. Rats under sodium pentobarbital anesthesia do not exhibit ascorbic acid depletion of the adrenal which normally follows exposure of animals to 3° C. for one hour (396). Sodium pentobarbital does not interfere with the ability of injected ACTH to deplete adrenal ascorbic acid. Furthermore, it would appear unlikely that sodium pentobarbital acts centrally to prevent the discharge of ACTH, since the anesthetic does not interfere with the ability of the adenohypophysis to dis-

charge ACTH in response to injection of histamine or epinephrine. It is known that barbiturate interferes with the activities of the temperature-regulating center, as shown by the failure of the anesthetized animal to shiver and to maintain normal body temperature during exposure to cold. Sayers and Sayers (396) suggest that the elimination of the cellular activity normally associated with homeostatic adjustment to cold, i.e., skeletal muscular activity, is responsible for the action of sodium pentobarbital in preventing increased adrenocortical activity. The suggestion is one of a number of possibilities; the final solution must await additional insight into the intracellular processes which 'utilize' the cortical hormone.

Metabolic Changes in Stress: Role of the Adrenal Cortex

The possible variations in the metabolic pattern of the organism are almost as numerous as the agents which may induce them. An evaluation of the metabolic functions of organs and organ systems which act alone or in combination to buffer distortions in the *milieu intérieur* would embrace almost every phase of physiology. From the standpoint of the topic at hand, two major questions arise. First, what are the metabolic changes accompanying stress which can be accounted for by hyperactivity of the adrenal cortex?. Second, what is the role of the adrenal cortex in correcting metabolic distortions induced by stress? At first thought it may seem obvious that, since hyperactivity of the adrenal cortex is an invariable accompaniment of stress, certain alterations in the metabolic pattern, characteristic of the actions of cortical hormone, may also be invariable accompaniments of stress. Certainly lymphocytopenia and eosinopenia are relatively consistent morphological changes which occur within the first few hours of exposure. However, the complexity of factors involved in most stresses makes it very difficult to make definitive assignments as to the cause of the majority of the alterations.

In a few instances our present concepts of the metabolic actions of the cortical hormone give a ready answer to its mode of action in restoring to normal one or more elements of the metabolic pattern. A number of specific examples may be enumerated, including the well known facts that insulin hypoglycemia is counteracted by mobilization of carbohydrate and inhibition of glucose oxidation, that blood glucose is maintained during fasting by gluconeogenesis, and that plasma sodium remains relatively normal during a dietary deficiency of sodium because of renal reabsorption of the cation. Although the known functions of the gland can explain the role of the adrenal cortex in a few specific instances, they fail to give us an understanding of the ubiquitous role of the gland in the homeostatic adjustment of the animal to innumerable stresses.

Current textbook concepts of the metabolic actions of cortical hormone are gross oversimplifications and impose serious limitations on our thinking. The concepts have been based on experiments in which the tissues of the organism responded to a plethora of cortical steroids; the actions of the cortical steroids under such circumstances may be more correctly considered pharmacological rather than physiological. Recent experiments of Ingle (199) have clearly demonstrated that the metabolic actions of the cortical steroids are more numerous and diverse than hitherto imagined. New functions have been uncovered by examining the actions of the cortical steroids

in circumstances in which tissues or organs are active and utilizing cortical hormone at an accelerated rate and where the dose of cortical steroid has been selected to meet the requirements of the organism. For example, cortical steroids may promote as well as inhibit glucose utilization (198, 207, 215), and produce anabolism as well as catabolism (212) depending upon the dose of steroid and the tissue needs. The impression is gained that the full expression of most bodily functions is dependent upon an appropriate supply of cortical hormone. Examples will be cited in the sections to follow.

Many workers have attempted to evaluate the role of the adrenal cortex in producing the metabolic alterations of stress by comparing the distortions which occur in intact and adrenalectomized animals exposed to the same conditions. Experiments of this nature have some merit but caution must be exercised in interpreting the results. Ingle (198, 199) has correctly emphasized the fact that lack of development of a particular metabolic change consequent upon removal of the adrenal cortex does not necessarily mean that the gland is causally concerned in that metabolic change in the intact animal. Rather, it may mean that the presence of cortical hormone is essential for the maintenance of the functional capacity of a particular tissue or organ to exhibit this change.

I. *Carbohydrate Metabolism.* Trauma in man, irrespective of its locus, is followed in many instances by glycosuria and reduced tolerance to glucose (464). Anoxia reduces the glucose tolerance of the dog (226, 446). Administration of thyroid will induce hyperglycemia and glucosuria in intact rats given a sub-diabetic dose of alloxan, whereas thyroid has no 'diabetogenic' action in adrenalectomized alloxan-treated rats (302). The obvious inference to be drawn from such data is that the diabetic-like state of stress is a result of the hyperactivity of the adrenal cortex, since one of the most characteristic actions of the cortical hormone is to inhibit glucose utilization. However, the following experimental data indicate that the adrenal cortex plays a supportive rather than a causal role in the phenomenon. Adrenalectomized alloxan-diabetic mice receiving a fixed daily amount of ACE exhibit as great a rise in fasting blood sugar after a course of typhoid injections as do intact alloxan-diabetic mice (471). The hyperglycemic reaction to trauma fails to occur in the absence of the adrenals, but a dose of adrenocortical extract, which does not elevate the blood sugar of non-traumatized normal rats, restores the hyperglycemia of trauma in adrenalectomized animals (423).

Special attention has been given to the derangements of carbohydrate metabolism in anoxia. Evans (117) demonstrated that the increase in blood sugar and liver glycogen which normally occurs after exposure to low atmospheric pressure does not occur in the absence of the adrenal cortex. Lewis and co-workers (255) confirmed and extended these studies. During the initial phase of anoxia, there appears to be an increased rate of utilization of carbohydrate; blood glucose is maintained (or even falls when the hypoxia is severe) while the store of liver glycogen is reduced; with time blood glucose and liver glycogen rise to greater than normal concentrations. Rats, rabbits and monkeys exhibit the changes just described, whereas the dog shows none of these alterations. Man likewise does not show a rise in fasting blood glucose or a reduced tolerance to glucose; however, the reduction in barometric

pressure employed in the human experiments was not as great as that for the other species. Bryan and Ricketts (43) have observed a slight and variable decrease in the tolerance of man to glucose after prolonged intermittent exposure to high altitude. According to Lewis *et al.* (255) ACE and cortisone, but not DCA, simulate the late changes of hypoxia (elevation in blood sugar and liver glycogen). Langley and Clarke (248) confirmed the fact that the increase in blood sugar and in liver glycogen which is characteristic of low atmospheric pressure requires the presence of the adrenal cortex. However, when a dose of ACE, which in itself does not affect carbohydrate levels, is administered as maintenance therapy to the adrenalectomized rat, it allows for the full development of the carbohydrate changes characteristic of hypoxia. These observations may be interpreted to mean that the adrenal cortex does not induce the changes in carbohydrate metabolism characteristic of hypoxia but rather that the changes are initiated by tissues whose functional activity requires the presence of cortical hormone.

Swingle *et al.* (453) have enumerated a great number of similarities between the derangement of patterns of metabolism in shock as it occurs in intact animals and in the crisis of adrenocortical insufficiency. They considered the blood sugar changes to be a point of discrepancy. However, it is now established that hypoglycemia characterizes advanced shock (507) in intact animals. The question may be legitimately asked whether the metabolic derangements of shock are a result of a deficiency of cortical hormone. Engel (114) states, "Since all types of stress are followed by evidence of increased protein catabolism and activation of the adrenal cortex in normal but not in adrenalectomized or hypophysectomized animals, whereas stress regularly results in hypoglycemia and death in the latter, it may be that an increased need for carbohydrate is the first effect of stress in general." According to Wilhelmi (507), the initial response to injury, an elevation in blood glucose with smaller increases in lactate and pyruvate, is due to reflex discharge of epinephrine; the hormone of the adrenal cortex is involved in the mobilization and catabolism of protein and in facilitating the formation of new carbohydrate. In fully developed shock, the blood sugar falls and there is a striking rise in the ratio of lactate to pyruvate. To quote Wilhelmi, "Since for equal energy output anaerobic glycolysis is several times as costly as the oxidation of carbohydrate, the combination of an increased energy demand with anoxia is principally responsible for the increased rate of utilization of carbohydrate in shock." It is exceedingly difficult to evaluate the role of the adrenal cortex even in this relatively simple aspect of the complex pattern of metabolic derangements which characterize anoxia. The conclusion that the hypoglycemia of advanced shock in the intact animal is a result of adrenocortical insufficiency could only be reached by *1*) accepting the gross oversimplification that cortical hormone action on carbohydrate metabolism is confined to mobilization of glucose and inhibition of its utilization, and *2*) neglecting the many factors other than the adrenal cortex which influence carbohydrate metabolism. Of interest in this connection is the demonstration by Ingle and Nezamis (206) that with certain doses of insulin and high rate of infusion of glucose the adrenalectomized eviscerated animal utilizes glucose more slowly than the non-adrenalectomized eviscerated animal. These same workers (207) also demonstrated in rats forced to exercise to the

point of fatigue that when glucose was infused at a rapid rate and when muscular responsiveness was lost, the blood glucose of the adrenalectomized animals rose sharply above that of the intact animals.

II. *Protein Metabolism.* Increased excretion of nitrogen is one of the most characteristic metabolic alterations which accompany the response of the organism to stress. In intact animals (217, 323) or in patients with normal function of the pituitary-adrenocortical system (355) there is an increased rate of excretion of nitrogen after trauma. Anoxia in the dog elevates urinary nitrogen (255). On the other hand, following adrenalectomy, trauma in the rat (42, 217, 323) or anoxia in the dog (255) fails to elevate urinary nitrogen. A patient with panhypopituitarism has been reported to have had a less than normal 'nitrogen catabolic response' to injury (41).

The A-V difference in amino acids of adrenalectomized dogs, in contrast to intact dogs, does not increase following hemorrhage (229) and adrenalectomy markedly inhibits the elevation in blood amino acids which follows evisceration in the rat (214). Experiments of this nature have been interpreted to mean that the adrenal cortex is largely responsible for the catabolism of protoplasm which accompanies injury.

However, recent investigations indicate that the role of the adrenal cortex is supportive rather than causal. Adrenalectomized rats maintained on a constant daily dose (4 ml.) of beef ACE exhibited as great an increase in urinary nitrogen after bone fracture as did intact animals (217). Adrenalectomy was followed by an actual increase in the urinary nitrogen excretion in partially depancreatized rats (211). Noble and Toby (323) demonstrated that amounts of ACE, which by themselves did not cause an increase in the urinary nitrogen excretion of untraumatized adrenalectomized rats, made it possible for adrenalectomized animals to excrete nitrogen at a rate comparable with that of intact rats in response to equal degrees of trauma. Furthermore, these same workers found that, although adrenalectomized rats exhibit no increase in urinary nitrogen in response to injury, they do accumulate non-protein nitrogen in the blood. The experiments indicate that hyperactivity of the adrenal cortex is not the causal mechanism in the nitrogen catabolism of injury. However, the presence of the gland is essential for the full metabolic response of tissue cells to injury. Recent observations of White and Dougherty (503) may be interpreted to mean that the catabolic action of thyroid requires the presence of adrenocortical hormone, although the authors themselves interpret their data to mean that the adrenal cortex has a stimulating action upon the thyroid gland.

As has been emphasized above, the metabolic functions of the cortical steroids may be quite different when examined in situations where the tissues are 'utilizing' the hormone at a rate approximately equal to the supply than when examined in circumstances where the tissues are exposed to an 'excess' of steroid. This is strikingly illustrated by experiments of Ingle and Prestrud (212) in which it is shown that certain doses of ACE will reduce urinary nitrogen excretion of adrenalectomized rats as compared to adrenalectomized controls maintained with 0.9 per cent sodium chloride solution (anabolic action), whereas larger doses produce an increase in the rate of excretion of nitrogen (catabolic action). It is of interest in this connection that ACTH administration is accompanied by positive nitrogen balance in children

with non-Addisonian hypoglycemia (294), and that ACE promotes liver regeneration in adrenalectomized partially hepatectomized rats (29).

Additional evidence has now appeared to support the thesis that the functional status of the target cells has an important bearing upon the character of the metabolic response produced by cortical steroid. According to Engel (114), "... the injection of the same dose of ACE may be followed by widely different responses depending on the internal metabolic environment of the organism at the time of treatment." Urea accumulation in nephrectomized rats treated with ACE plus insulin is three to four times greater than after ACE alone; glucose prevents both the hypoglycemia and the increase in urea formation seen after administration of insulin and ACE (114).

III. *Fat Metabolism.* Fat is mobilized and stored in the liver of the mouse following exposure to cold and after strenuous exercise; high temperature and low barometric pressure tend to cause a loss of fat from the liver (252). ACTH induces fat storage in the liver of the rat (258, 260) and mouse (252, 341). The data suggest that the adrenal cortex plays a role in the phenomenon of fat deposition in the liver in response to stress, although it is not yet definitely established whether it is a causal or supporting role. It has been reported (340) that, although the adrenals are essential to the action of the liver fat-mobilizing factor of the anterior pituitary in the mouse, the effect is not due to ACTH; neither is the effect mediated by the adrenal cortex. The interested reader is referred to the review by Ingle (191) for a more detailed discussion of the role of the adrenal cortex in fat metabolism.

IV. *Electrolyte Metabolism.* The inadequacies of our knowledge regarding the role of the cortical hormone in the regulation of electrolyte metabolism are becoming increasingly apparent with the availability of ACTH and cortisone for experimental studies. Considering these inadequacies and the fact that the gland is only one of a number of factors which influence electrolyte metabolism, it is not surprising to find that we are at a complete loss in most instances to evaluate the part played by the adrenal cortex in initiating or correcting distortions of electrolyte pattern which occur in stressful conditions.

The extensive and numerous observations on the actions of DCA represent but a single aspect of a complex problem; overemphasis has been attached to the fact that this synthetic steroid acts to induce sodium retention. Recently Forsham *et al.* (123) and Perera *et al.* (351) have demonstrated that cortisone can induce sodium retention in patients with Addison's disease. Furthermore, the secretion of the adrenal cortex maintains homeostasis of sodium in situations of dietary sodium excess as well as during dietary deficiency, whereas DCA administration continues to induce sodium retention regardless of the intake of the cation. The patient with untreated Addison's disease has considerable difficulty excreting sodium at a rate fast enough to prevent edema when he is presented with an excess of sodium (371), and the adrenalectomized dog has a reduced capacity to eliminate sodium during a high salt load (382). The elevation of plasma sodium produced by DCA in rats can be prevented by the simultaneous administration of ACTH or ACE (511). Woodbury *et al.* (511) have reached the tentative conclusion that the secretion of the adrenal cortex and the 11,17-oxysteroids can 'normalize' plasma sodium regardless of the direction

of the deviation from normal, whereas DCA acts unidirectionally to produce sodium retention under all circumstances. The concept assigns a much more important role to the 11,17-oxysteroids in the regulation of electrolytes than to DCA. There is cogent evidence for believing that DCA exerts only pharmacological effects in the body.

According to Selye (418), "A pronounced decrease in blood chlorides is one of the most constant changes of the shock phase. It gives way to marked hyperchloremia during the counter-shock phase." The changes are exaggerated and telescoped into a briefer period in adrenalectomized and hypophysectomized rats. It is indeed important, if confirmed, that these fluctuations in blood chloride represent an alteration in electrolyte metabolism common to all stresses.

A characteristic feature of adrenocortical insufficiency is an elevation of potassium concentration in the body fluids. A number of stresses, e.g., hemorrhagic shock, muscle crush, intestinal manipulation, intestinal obstruction, acute pancreatitis, pneumonia and surgical operation are characterized by an accumulation of potassium in extracellular fluid (see review by Harkins (162)). The adrenal cortex undoubtedly plays an important role under these circumstances in accelerating the renal excretion of potassium. However, as was pointed out in the case of other metabolites considered above, influence on potassium balance is only one of the many ways in which the gland may act to prolong or promote the survival of an organism subjected to stress.

A few studies have been directed toward the elucidation of the role of the adrenal cortex in both the development and prevention of the disturbances of electrolyte balance in anoxia. Lewis and co-workers (255) have demonstrated and Langley and Clarke (248) have confirmed that intact dogs exposed to low barometric pressure excrete more than normal quantities of sodium, potassium and chloride. Adrenalectomized dogs, maintained with DCA (255) or with ACE (248) and exposed to low atmospheric pressure, show an increased rate of excretion of potassium but not of sodium or chloride. In addition, Lewis *et al.* (255) found that one unexposed adrenalectomized dog, administered cortisone, had an increased rate of excretion of sodium and chloride but not of potassium. The experiments suggest that the sodiumphoresis and chloruresis in anoxia are mediated by 11,17-oxysteroids.

Disturbances in electrolyte balance of non-adrenal origin may simulate the changes which occur in adrenocortical insufficiency. For example, it has been suggested that the depletion of sodium and chloride accompanying porphyria might be on the basis of adrenocortical failure. However, Prunty (363) has applied various indices—eosinophil test, uric acid excretion, chemocorticoid (copper reducing lipids) excretion, gland size and histology—of adrenocortical function to four cases of porphyria and has reached the conclusion that the disordered electrolyte metabolism in this disease is not primarily adrenal in origin.

Resistance to Stress

The term 'resistance to stress,' as employed in this review, is defined as that capacity of an organism to maintain constancy of the internal environment and normality of bodily functions during exposure to stress. Degree of resistance to stress

may be measured in a number of ways, including mortality, maintenance of normal concentrations of metabolites in body fluids and maintenance or rate of restoration of individual functions.

Three aspects of the problem of resistance to stress will be considered: *1*) the striking reduction in resistance to stress which follows loss of adrenocortical function; *2*) the question of whether stress induces adrenocortical insufficiency and, its corollary, whether the resistance of the intact organism to stress can be increased by cortical steroid therapy; and *3*) the mechanism or mechanisms by means of which the secretion of the adrenal cortex increases the resistance of an animal to stress.

I. *Decreased Resistance to Stress after Adrenalectomy.* It has been firmly established that the adrenalectomized animal is extremely sensitive to the damaging effects of a great variety of agents and environmental changes. Replacement therapy will either partially or completely restore the resistance of the adrenalectomized animal to normal. The reader is referred to the article by Swingle and Remington (454) for a review of the literature on this subject. It is sufficient to point out here that the phenomenon is characterized by complete non-specificity in regard to the nature of the stressful agent.

II. *Does Stress Induce Hypocorticism?* It is at least theoretically possible that a state of hypocorticism exists when an animal is exposed to a very severe stress, because of *a*) inability of the pituitary-adrenocortical system, although normal, to produce cortical hormone in quantities sufficient to meet the accelerated demands of the tissues, *b*) 'exhaustion' of adrenocortical function, or *c*) 'exhaustion' of adrenohypophyseal function.

1) Does a Normal Pituitary-Adrenal System Ever Fail to Meet the Demands of the Tissues for Cortical Hormone in Severe Stress? There are certain instances in which the metabolic derangements of the intact organism exposed to stress resemble those which characterize adrenocortical insufficiency (see review by Swingle and Remington (454)). However, as pointed out in another section, a complexity of factors contribute to the metabolic pattern, and it would indeed be foolhardy to ascribe the changes of stress entirely to a deficiency of adrenocortical hormone.

Clear cut demonstration that cortical steroid therapy increases the resistance of the intact organism to stress would constitute fairly definite proof that the rate of adrenocortical hormone secretion is a limiting factor in survival. Swingle and Remington (454) have reviewed the pertinent literature through 1943. There were conflicting reports, but the overall picture presented by these authors may be summarized to the effect that cortical steroid therapy is of no benefit in improving the resistance of the intact organism. The majority of reports which have appeared since 1943 have also been negative.

Neither DCA nor ACE increases the survival of intact mice (27) or rats (204) from scald. ACE has no protective action in intact mice against the lethal effects of typhoid vaccine (254) or Type I (254, 472) or III (254) pneumococci. Large doses of ACE fail to affect survival but do improve ability to resist hypothermia in rats infected with *B. tularense* (357). Continuous subcutaneous and intravenous adminis-

tration of ACE fails to improve the work performance or to protect against collapse (hypoglycemic) of either fasted or non-fasted intact rats forced to undergo vigorous muscular activity (210).

Ellinger (109) claimed that daily administration of DCA increases the survival rate of irradiated mice, but Straube *et al.* (448) have been unable to confirm this claim. ACE is also ineffective against radiation, according to Straube and co-workers.

ACE has no effect upon the performance of men in a hot environment, and neither does the extract influence physiological or chemical indices of adrenocortical activity except for a slight effect in lowering the concentration of sodium and chloride and elevating the concentration of potassium in sweat, and causing an increased urinary excretion of potassium (303).

Anoxia is unique among stresses in regard to the number of confirming reports which have appeared on the therapeutic benefit of ACE in this condition. Thorn *et al.* (466) have demonstrated that Kendall's extract of beef adrenals reduces the mortality of intact rats exposed to low barometric pressure; DCA and cortisone are ineffective. The observations have been confirmed by Kottke *et al.* (240) who have shown that Kendall's, but neither Upjohn's nor Wilson's, extract of adrenal cortex increases the resistance of intact mice to hypoxia. However, Kottke *et al.* (240) demonstrated that even large doses of Kendall's extract are without effect upon the deterioration of coordination, vision and hearing that follows the exposure of the intact dog to acute hypoxia. Administration of ACTH increases the resistance of rats to low barometric pressure (257), an observation which suggests that adrenals, hypertrophied with ACTH prior to exposure, have a greater capacity to secrete cortical hormone than do normal glands.

Administration of glucose intraperitoneally is nearly as effective as Kendall's extract in increasing the resistance of intact mice to hypoxia (240), and a high carbohydrate diet increases the resistance of rats to low barometric pressure (37). It is tempting to conclude from these experiments that available carbohydrate is a limiting factor in survival from hypoxia and that cortical steroid exerts a beneficial effect by mobilizing glucose. Such a simple explanation is hardly adequate. It was pointed out in a previous section that disturbances in carbohydrate metabolism represent only one aspect of the complex pattern of events in anoxia. Furthermore, the lack of uniformity of therapeutic action among various extracts of the adrenal cortex suggests the possibility that an agent other than the cortical steroid is responsible for the effects reported.

Isolated findings of the therapeutic benefit of cortical steroid in intact animals have been reported. For example, large doses of ACE increase the survival rate of intact rats given peptone (192), increase the resistance of mice to pneumococcal infection (491), and prolong the survival of guinea pigs infected with *Clostridium welchii* (490).

2) 'EXHAUSTION' OF THE ADRENAL CORTEX. A number of authors speak of 'exhaustion' of the adrenal cortex with the implication that the gland can fail as a result of a period of intense secretory activity. Actually there is little justification for using the term, except possibly in situations of overwhelming infection (Waterhouse-Friderichsen syndrome); even here, it is more likely a problem of intoxication rather

than exhaustion. The marked depletion of sudanophilic substance, cholesterol and ascorbic acid in the adrenals in circumstances of severe stress has been the basis for the use of the descriptive term 'exhaustion' in many instances. The present state of our knowledge in regard to the relation of the concentration of certain constituents to the secretory activity of the gland does not yet justify the conclusion that a gland 'exhausted' of 'precursors' is impaired in its secretory activity, no matter how attractive or convenient such a conclusion may be. Furthermore, the numerous observations on the lack of therapeutic action of cortical steroid in intact animals exposed to stress suggest that failure of adrenocortical function is not a limiting factor in survival.

Diphtherial, meningococcal, staphylococcal and streptococcal toxins produce hyperemia, hemorrhage and necrosis of the adrenal cortex of the rabbit (264). Degenerative changes have been described in the adrenal cortex of guinea pigs given *Clostridium welchii* toxin (228). Focal necrosis of the adrenal cortex occurs in monkeys which succumb to malaria (144) and in the adrenals of man after infections (143). *B. tularense* induces a marked depletion of adrenal cholesterol in the rat (357). It is not unreasonable to suppose that the functional activity of the cells of the adrenal cortex is impaired by toxins and that a state of relative insufficiency of cortical hormone may be so induced. In man, the Waterhouse-Friderichsen syndrome, which is associated with acute fulminating infections, has many of the features of acute adrenocortical insufficiency and the adrenal cortex shows hemorrhagic and necrotic lesions. A number of reports (67, 158, 164, 168, 183, 218, 224, 315, 437, 444, 496, 513) have described recovery with the use of cortical steroid therapy, and Thorn *et al.* (467) recommend cortical steroid therapy in patients with overwhelming infection who develop shock. Unfortunately, the nature of the evidence in support of the notion that cortical hormone insufficiency contributes to the fatal outcome is not completely satisfactory, since small doses of cortical steroid were employed in many instances together with the fact that antibiotics occupied an important place in the therapeutic program.

Pantothenic acid deficiency decreases the degree of lymphocytopenia which normally occurs in rats after forced swimming (25 minutes in water at 25° C.) or after the administration of ACTH (104). Dumm *et al.* (104) interpret this to mean that pantothenic acid "plays an important role in maintaining adrenal cortical function." However, the experiments do not rule out the possibility that the phenomenon is due to a decreased sensitivity of the lymphocytes of the pantothenic acid-deficient animals to cortical hormone, or that the vitamin deficiency induces chronic hyperactivity of the adrenal cortex (the unstressed deficient animals have a lower lymphocyte count than unstressed normal animals) which in turn reduces the relative effectiveness of acute stress or ACTH in producing a lymphocytopenia.

In conclusion, the evidence is strongly suggestive, but by no means conclusive, that impairment of the secretory activity of the adrenal cortex is associated with acute fulminating toxic infections; this is more likely a direct cytotoxic disturbance rather than 'exhaustion.' The so-called 'exhaustion' from hyperactivity has no sound basis at present.

3) 'EXHAUSTION' OF THE ADENOHYPOPHYSIS. The possibility must be considered

that a deficiency of the secretory activity of the adrenal cortex may arise as a result of exhaustion of supplies and/or curtailment of synthesis of ACTH. Since ACTH is a protein it is reasonable to suspect that deficiency of amino acid precursors may limit the production of the hormone. Rate of excretion of biocorticoids by debilitated individuals subjected to an acute traumatic event increases slightly or not at all (478, 480) an observation which suggests that the adrenal cortex is less active in malnourished than in normal subjects in stressful situations. However, the interpretation of these studies is compromised by the fact that the pre-trauma rate of biocorticoid excretion is usually higher than normal. Selye and co-workers (88, 177, 306, 419, 428) have presented evidence which they interpret to mean that the adrenocorticotrophic activity of the pituitary is enhanced by high-protein intake and inhibited by low-protein intake. The work of Benua and Howard (25) is not in conformity with this conclusion.

Large stores of ACTH are present in the pituitaries of the following species: *1*) rats kept under optimal conditions; *2*) hogs killed at the slaughter house, and *3*) humans, as judged by assay of autopsy material from cases diagnosed as myocardial infarction, perforated peptic ulcer and erythroblastosis fetalis (45). It has been estimated that a single rat pituitary contains enough ACTH to produce a marked reduction (50% of maximal) in the ascorbic acid content of the adrenals of 200 rats. Furthermore, it has been shown by Merkin *et al.* (298) that, despite continued discharge of ACTH in response to scald, the pituitary content of ACTH is depleted not more than 50 per cent of normal. The observations strongly suggest that rate of discharge of ACTH is never a limiting factor in the ability of the pituitary-adrenocortical system of a normal animal to meet production requirements of an acute exigency.

The functional capacity of the pituitary-adrenocortical system of rats on a low-protein diet has been studied (392, 402). Malnourished rats have only slightly reduced stores of ACTH as compared to normal rats. Furthermore, the capacity to elaborate ACTH in response to scald is not appreciably altered in the malnourished rats as judged by the increase in weight of the adrenals and the reduction in adrenal cholesterol and ascorbic acid. Mice, restricted in caloric intake, show evidence of decreased gonadotrophic but increased adrenocorticotrophic activity of the pituitary (36). Whether these observations are true of man as well as the rat must await application of newer technics to the measurement of adrenocortical function in man. A pituitary[6] from an extremely cachectic individual with intestinal carcinoma was found to have a concentration of ACTH equal to that of the pituitaries of patients who were in a good nutritional state at the time of death.

The atrophy of the adrenal cortex and other pituitary target glands which follows chronic inanition in the rat has been described as a state of 'pseudohypophysectomy' by Mulinos and Pomerantz (309). Sayers *et al.* (402) are inclined to favor the view that the adrenal atrophy of chronic undernutrition is in response to a reduced need of the organism for cortical hormone rather than a defect in pituitary adrenocorticotrophic activity. When subjected to stress, animals on a diet restricted in calories discharge ACTH at an accelerated rate as evidenced by adrenal hypertrophy.

[6] Obtained from Dr. Thomas W. Burns.

The tentative conclusion may be reached that the survival of an animal with intact adrenals from exposure to an agent which severely taxes the resistive capacity of the organism is not limited by failure of the adrenal cortex to fulfill the needs of the tissues for cortical hormone. Hypoxia may be an exception to this general statement. The possibility must be kept in mind that inadequate doses of cortical steroid may have been employed in experiments designed to test the therapeutic effect of the substance in intact animals.

III. *Mode of Action of Cortical Hormone in Increasing Resistance to Stress.* The marked hypersensitivity to stress occasioned by loss of the adrenals has naturally aroused speculation as to the mechanism by means of which the secretion of the adrenal cortex confers resistance. A few of the suggested mechanisms will be considered. The inadequacies of attempts to explain the role of the adrenal cortex in resistance to stress on the basis of its metabolic actions have already been discussed.

1) Detoxification. Brown-Séquard originated the theory of detoxification (see Rolleston, 384) and it has persisted since his time in various modifications. The resistance of the animal to stress is believed to be related to the action of the cortical hormone in detoxifying noxious agents released from injured or metabolically deranged tissues.

Histamine has been indicted as an endogenous toxic agent by a number of investigators. The adrenalectomized animal has a less than normal ability to inactivate histamine, which ability can be restored to normal with ACE or DCA therapy (386). However, the observation does not constitute too strong an argument in favor of histamine as the toxic substance responsible for the collapse of the adrenalectomized animal upon exposure to stress, since it is only one of a great variety of toxic agents to which the animal with adrenocortical insufficiency is hypersensitive. Suggestive, but not conclusive evidence is that both blood (509) and tissue (387) histamine levels are elevated after adrenalectomy, presumably because histamine is inactivated less readily in the adrenalectomized than in the intact animal (386).

Histamine and other toxic amines undoubtedly contribute to the collapse and death of the adrenalectomized animal exposed to certain stresses, e.g., trauma, burns, and allergic reactions. However, if histamine were the toxic factor common to all stresses, then one would expect more impressive similarities than are known to occur between the signs and symptoms of histamine toxicity and of collapse in adrenocortical-insufficient animals exposed to stress.

2) Cardiovascular Dynamics. Swingle and Remington (454) have reviewed the evidence regarding the influence of cortical hormone upon the functional integrity of the cardiovascular system. Cardiac failure, loss of tone and responsiveness of arterioles, and atony and increased permeability of capillaries undoubtedly contribute to the collapse of the adrenalectomized animal subjected to measures which strain the cardiovascular system (56). However, although alterations in cardiovascular dynamics contribute to the collapse of the adrenocortical-insufficient animal, they can hardly be a major cause for the susceptibility of the adrenalectomized animal to various stresses.

3) Mobilization of Antibodies. According to White and Dougherty (501, 502), the lymphocytes serve as a storehouse for a portion of the globulin fraction of serum,

and the rate of release of this protein is under the control of ACTH. Any stimulus which augments the secretion of the adrenal cortex accentuates lymphocyte dissolution and globulin release. To quote White (499), "The demonstration that the lymphocyte is one of the end cells of adrenal cortical steroid action serves to integrate the role of the lymphocyte and the adrenal cortex in resistance. The control exerted by pituitary-adrenal cortical secretion over the contribution of the lymphocyte to immune globulin production is related to a specific type of resistance, immunity based on antibody formation. Whether this endocrine-lymphocyte relationship contributes to other types of resistance or immunity remains to be investigated."

Dougherty *et al.* (98) have presented evidence to indicate that the regulatory influence of the adrenal cortex over antibody mobilization offers an explanation for the anamnestic response, the elevation of antibody titer which occurs in response to a variety of non-specific stresses.

Eisen *et al.* (107), in agreement with Dougherty and White, found that a single injection of ACE increases the titer of antibody nitrogen in the serum by 30 per cent. However, Eisen and co-workers (107) could not demonstrate a prolonged effect of adrenocortical hormone upon the production or release of antibodies and gamma globulins. According to Murphy and Sturm (311), adrenalectomized rabbits produce antibodies far in excess of the amount produced by intact animals. Li and Reinhardt (259) could not produce an increase in the concentration of the gamma globulin fraction of plasma or duct lymph of rats with ACTH. In neither the cat nor the rabbit do single large doses of ACE alter the titers of immune bodies in the serum (462), nor does ACE influence the antibody content of cell-free lymph fluid in cats (64). ACE does not increase the immune response of intact mice to type I pneumococcal vaccine antigen (492). Milne and White (301) have shown that ACE increases total serum protein concentration but does not affect the relative distribution of serum proteins in the mouse. ACE induces a lymphopenia but no changes in serum globulin in the fowl (430). Fischel *et al.* (121), employing quantitative precipitin technics, failed to find experimental evidence in support of the concept that the anamnestic response is a result of hyperactivity of the adrenal cortex. Indeed, they raise doubts as to the existence of a non-specific anamnestic response and state that, "It is evident that until the question of a non-specific anamnestic response is resolved, the inferences drawn by other investigators as to the relationship of hormones and lymphocytes on circulating antibody must be in doubt."

Stimulation of the adrenal cortex of man with ACTH has not produced an increase in the titer of any one of a variety of antibodies (124, 178, 179, 394). Furthermore, ACTH has no influence upon the distribution of plasma proteins in normal subjects (124, 394). In pituitary insufficiency ACTH administration has been reported to elevate the concentration of plasma albumin and decrease the concentration of plasma α_2 globulin (124).

The evidence indicates that lysis of lymphocytes consequent to increased secret tory activity of the adrenal cortex is not an important source of antibody, and tha the action of the adrenal cortex in increasing the resistance of the organism to infections and bacterial toxins is apparently dependent in small measure at most upon the release of antibodies from lymphocytes. Perhaps the recent findings of Gordon

and Katsh (147) that the adrenal cortex exerts a regulatory influence upon the phagocytic capacity of the reticulo-endothelial system has an important bearing on the mechanism by means of which the adrenal cortex increases the resistance of the organism to a pathogen.

4) Ubiquity Concept. All the theories which have been advanced to explain the means by which the adrenal cortex increases resistance to stress have a common failing. They give a ready explanation to the actions of the adrenal cortex in increasing the resistance of the organism to a limited number of noxious agents or stressful conditions. On the other hand, they fail to explain the most important fact regarding the adrenal cortex and homeostasis, i.e., that the cortical hormone increases resistance to any and every type of stress. With each new action reported for cortical hormone it becomes increasingly evident that the adrenal cortex influences the functional activity of every tissue and organ in the body. Space does not permit even a brief enumeration of the literally hundreds of actions which have been ascribed to the adrenal cortex, but it would be difficult indeed to find a stress which did not involve some cellular function dependent upon the activity of the cortical hormone. Considered in the light of these facts it is not surprising to find a gland of such ubiquitous functions involved in the homeostatic adjustments of the organism to all stresses. It is no longer necessary to look about for an elusive 'resistance factor' but rather to consider the non-specificity of the adrenal cortex in stress as due to its ubiquitous role in cellular functions of the body. Such a point of view does not explain the mechanism by means of which the adrenal cortex acts in stress. Rather the concept focuses attention on a gap in our knowledge regarding the locus and mechanism of action of cortical hormone in the enzymatic processes of the cell. It is not unlikely that the varied actions which have been assigned the adrenal cortex are manifestations of a single fundamental role of the hormone in an enzymatic process common to all cells.

Adaptation to Stress

The word adaptation must have a restricted meaning in order to be of value in clarifying concepts in the field of homeostasis. Adaptation may be defined as that state of an organism characterized by increased resistance to stress through previous exposure to stress. In other words, it is that state wherein continued or intermittent exposure to stress induces changes which enable the organism more efficiently to counteract potential or actual alterations during subsequent exposure to stress. Adjustment to stress, on the other hand, should be used as a general term to characterize those types of reactions which concern the mobilization of homeostatic mechanisms which are available to the organism in the presence or absence of any previous exposure. Specific or non-crossed adaptation refers to adaptation acquired through repeated exposure to the same agent; non-specific or crossed adaptation refers to adaptation to a particular stress acquired through exposure to a different stress.

A striking example of adaptation is the development of antibodies to bacteria, viruses or toxins. Continued exposure to low barometric pressure induces an increase in the number of circulating red blood cells. Work hypertrophy of muscle may be

considered to be an example of adaptation. Tolerance to drugs must be accompanied in many instances by the acquisition of unique changes in certain organs. Tolerance through continued exposure may actually be of such a nature that withdrawal of the drug represents a stress. The morphine addict exhibits severe and dramatic physical changes when the drug is suddenly withdrawn.

What role does the adrenal cortex play in the acquisition of adaptation to stress? Langley and Clarke (248) have shown that there is a marked increase in the requirement of adrenalectomized rats for cortical steroid during the initial period of exposure to low atmospheric pressure, but despite continued exposure the steroid requirement returns to pre-exposure levels. The results of studies of intact rats by Darrow and Sarason (73) and Dalton *et al.* (68) are in agreement with this conclusion. Depletion of the sudanophilic substance of the adrenal cortex occurs during the first two days of exposure to low barometric pressure, followed by restoration of the normal pattern of lipids by the seventh day; rats fed a normal diet show no significant change in adrenal size during the course of the experiment; rats fed a diet low in potassium exhibit adrenal hypertrophy on the second day followed by return to normal weight of the gland by the seventh day (73). Exposure of rats to low atmospheric pressure induces adrenal hypertrophy. However, repeated exposure is in time accompanied by a return toward normal size of the glands, associated with normal amounts and distribution of lipid (68). Apparently adaptation is characterized by a reduction in the requirement of the organism for cortical hormone.

Adaptation can be acquired in the absence of the adrenal cortex. Adrenalectomized rats develop increased resistance to the traumatic effects of rotation in the Noble-Collip drum (321) after repeated 'drummings.' However, a greater period of time is required for the attainment of a particular degree of inurement in adrenalectomized than in intact animals. Additional quantitative experimentation is required before final conclusions can be reached in regard to the role of the adrenal cortex in the actual process of adaptation. Tentatively it may be concluded that adaptation is a process concerned chiefly with changes in organs or systems of homeostasis and that the adrenal cortex is involved secondarily insofar as the secretion of the gland is essential for the full working capacity of the buffer organs.

Selye (418) has arrived at some rather general conclusions regarding adaptation on the basis of observations made in his own and other laboratories. He states, "Probably the most important functional change, which occurs in the course of the general adaptation syndrome, is the change in resistance to damaging agents. It has been found that if an animal is continuously treated with the same, sublethal, daily dose of one alarming stimulus, its resistance to an additional minimum lethal dose of that same stimulus decreases during the shock phase and gradually returns to, or above normal, in the counter-shock phase of the alarm reaction. During the subsequent stage of resistance, a considerable amount of specific adaptation to this same alarming stimulus becomes evident; yet if exposure is continued over a period of many weeks or months, eventually this acquired adaptation breaks down. Apparently, even a fully inured organism cannot indefinitely maintain its adaptation when continuously exposed to a great amount of stress. It is this observation which led

to the concept of 'adaptation energy.' Apparently, under the influence of continuous adaptive work, the adaptability or 'adaptation energy' of the organism is eventually exhausted. The time at which this breakdown of adaptation occurs, is referred to as the 'stage of exhaustion.'

"The non-specific resistance is likewise characteristically influenced by the general adaptation syndrome. That is to say, while an animal acquires resistance to a certain agent, its resistance to other agents is also altered. During the shock phase the non-specific resistance decreases, even more than the specific resistance. In the counter-shock phase, on the other hand, there is an increase in the ability of the animal to withstand various types of stress, not only that to which it had been previously exposed. Yet this 'non-specific' or 'crossed-resistance,' is never as great as the specific resistance to the particular agent with which the alarm reaction had been produced."

Gasic (132) has shown that mice given repeated injections of epinephrine develop resistance to epinephrine (specific adaptation) but not to formalin. The specific resistance which develops to formalin is of a local character; increased resistance to repeated injections of formalin does not occur unless the injections are confined to the same site. Furthermore, non-specific resistance to epinephrine acquired by repeated injection of formalin is of a local nature. Noble and Toby (322) could obtain no conclusive evidence that repeated induction of limb ischemia by clamping induces specific resistance to limb ischemia or non-specific resistance to drumming.

A carefully controlled quantitative study by Thatcher and Radike (463) indicates that the adrenal cortex is responsible for the acquisition of crossed resistance. Rats could be made to tolerate otherwise highly lethal doses of potassium salts by repeated injection of sublethal doses of potassium (specific adaptation). Increased resistance to potassium could be developed by repeated application of any one of a number of agents other than potassium (non-specific adaptation). However, the increase in resistance conferred by non-specific adaptation was slight compared to that conferred upon the animal by specific adaptation. Administration of either ACE or DCA produced protection of the same order of magnitude as that afforded by non-specific adaptation.

It appears that the adrenal cortex is not essential for the development of adaptation although the presence of the gland makes it possible for the animal to adapt more quickly. Specific adaptation, acquired by alterations in certain buffer organs, makes a much greater contribution to resistance than does non-specific adaptation.

Are Pathological Changes Induced by Hyperactivity of the Adrenal Cortex Which Accompanies Stress?

A possibility which merits serious consideration is that pathological changes may develop in the organism, not only by the direct action of mechanical forces, bacteria or toxins but also by hyperactivity of the adrenal cortex, an essential accompaniment of the normal defense reaction of the organism.

I. '*Adaptation Syndrome.*' Selye (418) has described the pattern of alterations which may occur upon exposure of an animal to stress under the term 'general ad-

aptation syndrome'; the syndrome is divided into three stages, the alarm reaction, the stage of resistance, and the stage of exhaustion. During the alarm reaction, tissue catabolism occurs with involution of the thymus and lymphatic tissue, lymphopenia, increased number of circulating polymorphonuclear leucocytes, erosions and ulcers in the gastrointestinal tract and involution of the pancreas. The blood sugar is elevated initially; it then falls and may reach hypoglycemic levels. According to Selye, blood concentrations of non-protein-nitrogen and potassium rise; the concentration of blood chloride decreases in the alarm reaction. The thymus and lymph nodes fail to involute during the alarm reaction in the adrenalectomized or hypophysectomized animal; all other signs of the alarm reaction are particularly severe in these animals.

According to Selye (418), most morphologic and biochemical changes of the 'alarm reaction' disappear during the stage of resistance and indeed, in some cases, the direction of the deviation from the normal is reversed, e.g., hypochloremia during the alarm reaction, hyperchloremia during the stage of resistance. Selye (418) quotes Masson (285) in this regard as follows: "In rats continuously treated with various damaging agents (exposure to cold, forced muscular exercise, formaldehyde injections), there develops an initial period of hypoglycemia (alarm reaction) followed by a second period of hyperglycemia after a certain degree of adaptation is acquired (stage of resistance)." Regarding the stage of exhaustion Selye (418) says, "If exposure to abnormal conditions continues adaptation wears out, the lesions characteristic of the alarm reaction (involution of lymphatic structures, adrenal enlargement, gastrointestinal ulcers) reappear and the stage of exhaustion develops during which further resistance becomes impossible."

To quote Selye further (418), "—during the stage of resistance and the stage of exhaustion of the general adaptation syndrome, nephrosclerosis or even acute nephritis occurs, especially in animals sensitized by unilateral nephrectomy and a high sodium intake. These renal changes are accompanied by a malignant type of hypertension, proteinuria and sometimes, widespread periarteritis nodosa with cardiac nodules which resemble the Aschoff bodies of rheumatic fever. —all experimental observations are most readily compatible with the view that during the general adaptation syndrome, certain hormones of the anterior pituitary and adrenal cortex are produced in excessive amounts in order to increase resistance; this defensive endocrine response is valuable in as much as it facilitates adaptation to stress (e.g., infections, intoxications, nervous commotions, cold, *etc.*), but the resulting endogenous hormone overdosage may become the cause of certain cardiovascular, renal and joint diseases. Thus developed the concept that many of the most common maladies of man are 'diseases of adaptation,' that is to say, the by-products of abnormal adaptive reactions to stress."

The reader is referred to the original papers of Selye (413, 418) for detailed descriptions of the gross and histological changes which accompany stress. They represent valuable contributions to the sum total of knowledge. On the other hand, the interpretations of the phenomena, although skillfully threaded together into an attractive and comprehensive concept, are open to considerable criticism.

II. *'Excessive' Production of Adrenocortical Hormone.* Before considering the

experimental data, certain points of view regarding adrenocortical hyperactivity must be clarified. The statements that "—during the general adaptation syndrome, certain hormones of the anterior pituitary and adrenal cortex are produced in excessive amounts—" and that "—the resulting endogenous hormone overdosage may become the cause of certain cardiovascular, renal and joint diseases" convey the meaning that the secretion of the adrenal cortex is produced in quantities greater than the requirements of the tissues; as a result the organism is flooded with an excess and signs of overdosage occur. Everyone is agreed that the adrenal cortex is hyperactive in stress and that cortical hormone production is greater than under optimal conditions. However, this does not mean that production is 'excessive' or that symptoms of 'overdosage' occur; rather it means that production has been accelerated to meet increased needs. Selye fails to distinguish between cortical hormone production by the adrenal cortex and the status of the tissues of the organism in regard to cortical hormone requirement. Adrenocortical activity has no necessary relation to the status of the tissue cells of the organism; it is possible for the tissues to be in a state of eucorticism, hypercorticism or hypocorticism with a hyperactive gland depending upon the requirement of the tissue cells for hormone.

The elucidation of the mechanism of regulation of adrenocortical secretory activity, considered in detail in a previous section, has an important bearing on the problem at hand. The impression is gained from Selye's reports that the adrenal cortex is slow in getting started following exposure to stress; but once having attained hyperactivity, it has a certain momentum which leads to excessive production of cortical hormone in the stage of resistance. He says (418), "—the adrenals play an important part during the alarm reaction and secrete adrenalin immediately after exposure, even before the shock phase is evident, while corticoid secretion is a counter-shock phenomenon." As stated above he considers the resistance stage to be accompanied by excessive production of cortical hormone. Contrary to this point of view, which implies that a considerable degree of sluggishness obtains in the pituitary-adrenal system, is the evidence, cited in previous sections of this paper, which indicates that the system is very dynamic. It is doubtful that the inherent inertia of the system is great enough to allow fluctuations to hypo- or hypercorticism to persist for more than an hour; on the whole, a fairly uniform state of eucorticism is maintained.

However, regardless of the point of view—'endogenous hormone overdosage' or accelerated production to maintain eucorticism—the practical question arises whether the hyperactivity of the adrenal cortex is responsible for the production of pathological changes in the organism. Selye (417) speaks of "—the development of overdosage symptoms due to chronic intoxication by the endogenously produced cortical hormones." Evidence pertinent to this thesis—the production of pathological and toxicological changes in animals by administration of DCA and anterior pituitary extracts, the pathology of Cushing's syndrome, the production of 'diseases of adaptation' in experimental animals by exposure to stress, and the nature of certain diseases of man classified by Selye as 'diseases of adaptation'—will now be analyzed.

III. *DCA Toxicity.* Selye and co-workers (415, 418, 426, 427, 429, 470) have

described pathological changes in the heart, blood vessels, kidney, brain and joints following the administration of DCA. In rats, unilateral nephrectomy and a high salt intake are necessary adjuncts to DCA treatment in order to produce the changes with a fair degree of uniformity. Cardiac lesions (similar to those seen in rheumatic endocarditis in man and hence called by Selye Aschoff-like nodules), periarteritis nodosa, polyarthritis (resembling the joint changes in rheumatic fever) and renal changes ('nephrosclerosis') have been observed in the treated rats. In the chicken (427), administration of excessive quantities of sodium chloride in the absence of DCA induces edema and nephrosclerosis; DCA in the chicken elicits similar effects even on a normal sodium intake (415). Pirozynski and Akert (362) produced arthritis, periarteritis nodosa and interstitial myocarditis in rats given a high salt diet and DCA.

Darrow *et al.* (72, 105, 300), working with rats, observed that a low-potassium intake induces cardiac pathology and renal hypertrophy similar to that produced by DCA. Addition of potassium chloride to the drinking water prevented the development of cardiac (72) and renal changes (105) in DCA-treated rats as well as in animals given a diet deficient in potassium.

DCA aggravates the renal damage produced in rats by cytotoxic serum (232) or by anti-placenta serum (266). Degenerative lesions were observed in the cerebral arteries of a patient with Addison's disease who had been given large doses of DCA just prior to death; similar but not identical lesions were produced in dogs, rats and rabbits by DCA treatment (125). Liver damage occurs in dogs given large doses of DCA and addition of salt to the diet enhances the hepatotoxic action of the steroid (435).

The similarity between the pathological changes produced in experimental animals by DCA treatment to those seen in certain disorders of man (rheumatic fever, rheumatoid arthritis, periarteritis nodosa) has impressed Selye and led him to the conclusion that hyperactivity of the adrenal cortex is an etiological factor in the development of these diseases in man.

However, it should be mentioned that it is difficult uniformly to reproduce pathological changes in experimental animals by DCA administration. DCA has been observed to cause slight damage to the renal tubules and to have no influence upon the renal vascular system or the glomeruli (23). Prolonged daily administration of DCA did not induce pathological changes in the heart of the rat according to one group of investigators (48). In Selye's own reports it is obvious that the types and degree of pathology vary from one experiment to the next, despite what appears to be uniform treatment. In order to increase the reproducibility of the phenomenon, rats must be given a high salt intake in addition to DCA and be sensitized by unilateral nephrectomy. Darrow and Sarason (73) have found that the tendency of DCA to produce cardiac lesions is aggravated by exposure to low atmospheric pressure. Harrison (166) attributes the lesions described by Selye to infection, alone or in combination with cold. Rats with pneumonia and exposed to cold develop cardiac lesions and nephrosclerosis whether treated with DCA or not; the pathology is actually less severe in the DCA-treated rats. Unfortunately, Harrison employed only

a small number of animals. The observations of Pemberton *et al.* (342) further attest to the variability of the phenomenon; arthritic-like lesions develop in both untreated and DCA-treated rats. The impression is gained that DCA must act in combination with as yet unknown factors in the production of pathological lesions in experimental animals.

IV. *Toxicity of ACE and 11,17-Oxysteroids.* Neither ACE nor 11,17-oxysteroids (cortisone-like compounds) have been shown to produce pathological changes similar to those induced by DCA. A possible exception is the case of one rat given ACE which according to Darrow and Miller (72) developed changes in muscle of a less severe degree than those produced by DCA. Cortisone does not prevent nor does it exacerbate the development of experimental cytotoxic serum nephritis in the rat; cortisone does induce moderate hypertension in intact nephritic rats and striking hypertension in adrenalectomized nephritic rats (231). With the availability of cortisone, more data on this important problem will undoubtedly appear in the near future. In the meantime, if it is assumed that hypertension falls into a different category from the pathology of the collagen diseases, then DCA is unique among the various types of cortical steroid preparations. Selye (417) is aware of this and he says "—nephrosclerosis, renal hypertension, periarteritis nodosa and perhaps even some of the so-called rheumatic conditions are 'diseases of adaptation,' due to an excessive or otherwise abnormal (*preferential formation of salt-active corticoids*) adrenal cortical response to non-specific damage." (Italics are the reviewer's.)

V. *Toxicity of Anterior Pituitary Extracts and Purified ACTH.* Hall *et al.* (160) have been able to produce nephrosclerosis and cardiac lesions in intact, but not in adrenalectomized rats, by the administration of a lyophilized extract of anterior pituitary tissue. A high protein diet is an essential factor in the development of the pathology (88). Ingle (199) has confirmed these observations. Unilaterally nephrectomized immature female rats on a high-sodium chloride, high-protein diet develop renal and myocardial pathology when given large quantities of lyophilized anterior pituitary powder; adrenalectomized animals given 4 ml. of ACE per day do not develop lesions under the same conditions of treatment.

The pituitary powder employed by Hall and co-workers (160) is extremely toxic, a factor which must be taken into account in arriving at an explanation for the development of the pathology. Thirteen of 20 intact and 1 of 10 adrenalectomized rats given the powder were alive on the 29th day of injection; ACE replacement therapy increased survival to 7 of 20 in the adrenalectomized group.

Stability studies in the reviewer's laboratory (140) indicate that the method of preparing lyophilized extract of anterior pituitary results in the loss of the greater portion of the adrenocorticotrophic hormone activity. It is quite unlikely that the factor which induces pathology is ACTH.

Ingle (199) makes the following comments regarding his own experiments with pituitary extract. Adult female rats do not develop pathological changes when subjected to the same treatment as the immature animals; pituitary-treated rats ate and grew more than controls; the site of injection of the powder is heavily contam-

inated with bacteria[7]; lyophilized powder of pituitary is a hormone complex. Ingle reaches the tentative conclusion that the adrenal cortex is a supportive but not a causative factor in the development of nephrosclerosis, cardiac and joint lesions.

Simpson and co-workers (434) could detect no change in the size or histology of the kidney of the rats given a purified preparation of ACTH for short periods (24–72 hours) or for long periods (4th–79th day of life). Dougherty (95) has injected mice with a purified preparation of ACTH for as long as 38 weeks. ACTH induces hypertrophy of the juxtaglomerular apparatus of the kidney; renal arteriolar degeneration and tubular casts are more extensive and more frequent in the ACTH-treated animals than in the controls. ACTH administration is accompanied by a proteinuria in the female mice which normally excrete only a trace of protein in the urine. Arteriosclerotic changes occur in the coronary and testicular arteries of animals treated with ACTH for longer than 18 weeks. No Aschoff nodules, periarteritis nodosa or increase in heart size were observed in any of the mice.

Woodbury *et al.* (512) have determined the influence of purified ACTH upon the degree of pathological change which occurs in unilaterally nephrectomized rats given sodium chloride and DCA. The trophin given simultaneously with DCA actually counteracts the toxic actions of DCA.

VI. *Pathology of Cushing's Syndrome.* In Cushing's syndrome there is a high incidence of nephritis and arteriosclerosis in adults (108) and of kidney damage in children (54). However, the reviewer is unaware of a report to the effect that Aschoff-like nodules, periarteritis nodosa or rheumatoid arthritis occur in Cushing's syndrome.

VII. *Production of 'Diseases of Adaptation' in Experimental Animals.* Selye (416) exposed a group of unilaterally nephrectomized rats for about two months to cold (+2° to +4°C.); another group was injected repeatedly during this period with formalin, and a third group was exercised vigorously. Six of eight rats exposed to cold and receiving sodium chloride in the drinking water developed hypertension and nephrosclerosis. Two of eight given tap water to drink and exposed to cold and two of each of the formalin and exercised groups given sodium chloride to drink developed hypertension and nephrosclerosis. "Aschoff bodies in the heart muscles, periarteritis of the intestinal vessels or arthritic changes, such as are produced by chronic overdosage with DCA, were not observed in any of the animals of this series." It would appear from these studies that cold, formalin or exercise alone or in combination with high salt intake is not capable of reproducing the changes induced by DCA, although Selye (418) states in a later paper, without presentation of data, that "—partial kidney insufficiency produced by unilateral nephrectomy sensitizes animals to the development of nephrosclerosis, nephritis, hypertension and *periarteritis nodosa*, following chronic exposure to damaging agents, such as cold." (Italics are the reviewer's.) Selye has not presented convincing evidence that chronic exposure of animals to stress (uncomplicated by DCA administration) induces pathological changes characteristic of the collagen diseases of man (periarteritis nodosa, interstitial myocarditis and arthritis).

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[7] Ingle (personal communication) has found that pituitary powders relatively free from bacteria do not induce pathological changes in immature rats.

VIII. *Adrenal Cortex and Essential Hypertension.* The etiology of essential hypertension remains obscure although renal ischemia in experimental animals results in the development of a hypertensive state which has many resemblances to the disease as it occurs in man (see review by Shorr (433)). The fact that hypertension is almost an invariable accompaniment of Cushing's syndrome, that hypertension can be induced by DCA-treatment and that renal hypertension is not maintained in the absence of either the adrenal cortex or adenohypophysis indicates that the adrenal cortex plays a definite role. Sapeika (388), after reviewing the literature on the subject, goes so far as to conclude that the adrenal cortex plays a major role in arterial hypertension.

Selye (417) observed that the administration of large doses of DCA produces hypertension in the rat, cat, guinea pig, mouse, dog and monkey; unilateral nephrectomy and sodium chloride sensitize the animals to the hypertensive action of DCA. Green *et al.* (151) have produced hypertension in rats with DCA plus sodium chloride and with DCA alone and suggest "—the possibility of arresting human hypertensive disease either by removal of the adrenal cortex or by nullification of the activity of its salt-retaining steroids." DCA, in a dose which had no effect in normal rats, induced hypertension in rats treated with cytotoxic serum (232) or anti-placenta serum (266). Friedman *et al.* (130) claim to have produced hypertension in rats by DCA administration, although the elevation in blood pressure which they observed was hardly great enough to be considered a hypertensive change.

A combination of DCA and high sodium chloride intake elevates the blood pressure of hypertensive subjects (348, 349). DCA induces a rise in blood pressure in normotensive subjects, but the rise in blood pressure is slower in onset than in hypertensive patients (350). Goldman and Schroeder (145) claim that the intravenous administration of DCA, but not of a number of related steroids, produces a prolonged elevation in blood pressure in hypertensive subjects. However, the rises in blood pressure, when they occur, are relatively small. Luft and Sjögren (275) have found that relatively small doses of DCA produce edema or hypertension in subjects with Addison's disease complicated with organic renal disease; the same doses have no effect in patients with Addison's disease uncomplicated with organic renal disease.

It is true that DCA produces hypertension in certain circumstances. However, large doses of DCA combined with a high-salt intake have failed to produce hypertension in dogs (449) and rats (23). According to Perera (344), the daily injection of DCA into 5 patients with uncomplicated hypertensive vascular disease for periods of 30 days or more produced a rise in blood pressure in all cases, but the elevation was sustained in only 3 of the 5 subjects.

If hypertension is due to hyperactivity of the adrenal cortex, then it follows that the indices of the rate of secretion of the gland should reveal this accelerated activity. In a review of the changes in the adrenal cortex associated with hypertension in man, Sapeika (388) reaches the conclusion that, "There is considerable anatomic evidence that hyperplasia or adenomatous change of the adrenal cortex is

a frequent concomitant of arterial hypertension, the incidence of such morphologic change being greater than could be accounted for by the factor of age alone." He goes on to say, however, that "—there may be no evidence of cortical hyperplasia, the size of the adrenals being within normal range; adenomatous hyperplasia also occurs in the absence of hypertension." The interested reader may refer to Sapeika's review for an extensive citation of the literature.

Hypertensive subjects have been reported to have an exaggerated eosinopenia in response to insulin and epinephrine (249). However, cholesterol is present in relatively high concentration in the adrenals of hypertensive patients (2, 383), a change which is indicative of relative inactivity, according to evidence set forth in another section of this review. Rogers and Williams (383) suggest that the change is "—the result of repeated cycles of adrenal stimulation and depletion followed by 'involution' and storage of excessive amounts of cholesterol." Only two of eight cases of hypertension examined by Tompsett and Oastler (473) had a urinary excretion of chemocorticoids (copper reducing lipids) exceeding the upper limit of normal (table 1). Frances Selye (412) reported an elevation of glycogenic biocorticoids in 4 of 10 male hypertensive subjects; 2 of 8 female hypertensive subjects had an elevated excretion but were suspected of having Cushing's syndrome (table 1). It is obvious that adrenocortical hyperactivity is by no means an invariable accompaniment of hypertensive disease.

Is there a distortion of electrolyte pattern in hypertension compatible with the notion that there is a 'preferential formation of salt-active corticoids' by the adrenal cortex? In this connection it is important to note that the concentration of serum sodium is normal in hypertensive subjects (227, 344). Frances Selye (412) reported that the ratio of serum concentration of sodium to chloride was elevated in about one third of a group of hypertensive subjects. Unfortunately, the actual values of the serum concentrations of sodium and chloride were not presented, which makes it difficult to evaluate critically the observed changes. According to Perera and Blood (347), sodium restriction is followed by a loss in weight and increased urine output in normal but not in hypertensive subjects.

The slight and inconsistent changes in electrolyte balance which have been observed in hypertensive subjects may be due to renal dysfunction rather than adrenocortical hyperactivity. Electrolyte balance studies combined with careful evaluation of renal function are required to clarify the problem. The evidence available at present does not justify the conclusion that hypertension in man is accompanied by an excessive secretion of a 'salt-active' hormone from the adrenal cortex.

Experimental and clinical work on the effect of sodium restriction on hypertension has been advanced in support of the thesis that hypertension is due to 'overactivity of a salt-active secretion' of the adrenal cortex. Grollman and Harrison (156) prolonged the survival period and lowered the blood pressure of hypertensive rats by drastically restricting their sodium intake, and Schwartz (408) reduced the average mean arterial pressure of hypertensive dogs from 182 to 138 mm. Hg by placing the animals on the low-salt rice diet of Kempner.

Salt restriction has been claimed to be of therapeutic value in hypertension in

man (44, 85, 157). However, Schroeder (407), in a critical review of the subject, is unwilling to accept the claims as proven because of the lack of adequate controls in the reported studies; Schroeder himself could not detect a significant effect of low-salt intake on blood pressure except in an occasional patient. Landowne *et al.* (246, 247) observed a very small reduction in the blood pressure of hypertensive subjects following the induction of salt restriction. Futhermore, the reduction in blood pressure which follows salt restriction in hypertension in man is not great enough to implicate a 'salt-active' steroid as an etiologic agent on the basis of the known effects of such restriction upon the hypertensive action of DCA in experimental animals.

Adrenalectomy (31, 134, 142, 331) and hypophysectomy (13) reduce the blood pressure of animals with renal hypertension to normotensive or nearly normotensive levels. The elevation in blood pressure induced by auditory stimulation in rats is not maintained in the absence of the adrenals (288). In a case of hypertension associated with Addison's disease, the blood pressure remained elevated during treatment with DCA; the blood pressure dropped to normal limits when DCA was withdrawn (343). Reduction of blood pressure to normotensive levels has followed adrenalectomy in a hypertensive subject (152). The observations do not necessarily mean that the adrenal cortex is causally concerned in the production of hypertension. The more likely explanation is that the adrenal cortex is essential for the maintenance of hypertension, but is not an inciting agent (142, 199, 332). Perera (345) concludes that "—no data have been presented thus far which establish the adrenal cortex as playing a primary causative role in hypertensive disease in man." Cortical hormone may be indirectly involved in one or more ways. For example, it may be required for the maintenance of vascular tone, the production of VEM (vasoexcitor material) by the kidney (517, 518) or the production of hypertensinogen by the liver (135, 187). In the absence of definite data another possibility must be entertained; arteriolar tissues of hypertensive subjects may be hypersensitive to cortical hormone and contract abnormally in the presence of normal titers of cortical hormone.

IX. *Allergic Basis of Rheumatic Heart Lesions and Periarteritis Nodosa.* Rich and Gregory (377, 378, 379) have presented evidence in support of the concept that rheumatic heart lesions and periarteritis nodosa are pathological manifestations of allergic phenomena. Rabbits sensitized to sterile horse serum show evidence of serum sickness and develop cardiac lesions strikingly similar to those characteristic of rheumatic carditis in man. "A careful study of these lesions shows that their nature and situation correspond in fundamental ways to those of the peculiar tissue reaction characteristic of rheumatic carditis, viz., focal edema of the connective tissue with swelling and degeneration of the collagen fibrils; inflammatory pericardial, valvular and mural endocardial infiltrations, the latter localized particularly at and near the insertions of the valves; and focal nodular accumulations of cells situated in the endocardium, in the valves, and adjacent to the arteries in the connective tissue septa of the myocardium. These cell accumulations in the experimental animals contain, in addition to macrophages and a few lymphocytes, the peculiar types of cells

familiar in the Aschoff body and, as in the Aschoff body, the cells may be found collected about the periphery of a focus of degenerated collagen. This is particularly evident in the substance of the valves and in the mural endocardium near the valvular attachments" (378).

Rich and Gregory (377) have also induced periarteritis nodosa in rabbits by the administration of sterile horse serum. "Examination of the histological sections showed beautiful arterial lesions, typical of periarteritis nodosa. Every stage of the process encountered in human periarteritis nodosa could be found in the various rabbits, and in the majority of the animals all stages were present in different arteries in the same rabbit."

Several workers (66, 126, 129, 184, 261, 293, 380) have confirmed the work of Rich and Gregory. Streptococcus toxin also produces the widespread lesions of collagenous disease in the rabbit (66).

Recent clinical reports that ACTH and cortisone are of therapeutic value in the treatment of collagen diseases, a group of disorders suspected of being manifestations of hypersensitivity phenomena, are of considerable interest in this connection. The demonstration that ACTH and cortisone can prevent the development of rheumatic heart lesions and periarteritis in horse-serum-treated rabbits would contribute additional support to the allergic theory of the collagen diseases in man.

X. *Deficiency of 11,17-Oxysteroids Induced by DCA.* According to Cheng and Sayers (50), DCA treatment leads to a state of hormone imbalance characterized by an excess of DCA (exogenous steroid) and a deficiency of endogenous 11,17-oxysteroid production. To quote these authors, "The role of the deficiency state as well as that of the excess DCA must be considered in arriving at conclusions regarding the etiological role of DCA in the production of pathological conditions of the cardiovascular system." The pathology produced by DCA in experimental animals may be due to a lowering of 'resistance' of the tissues as a result of a deficiency of 11,17-oxysteroids. In conformity with this prediction are the experiments of Woodbury *et al.* (512) which indicate that simultaneous administration of ACTH and DCA inhibits the development of pathological changes induced by DCA alone. In two recent reports it appears that Selye (420, 421) has now also adopted the view that DCA creates a condition of hypocorticism.

XI. *Therapy of Collagen Diseases with Cortisone and ACTH.* The recent clinical discoveries (175, 176, 465) that ACTH and cortisone are of benefit in the treatment of the collagen diseases mark the beginning of a new era in the therapeutic application of cortical steroids. Unfortunately, the adrenal physiologist is at a loss to give a rational basis for these empirical discoveries.

The findings are at odds with the thesis that the collagen diseases are induced by hyperactivity of the adrenal cortex. The modification of the 'excessive production' concept to the 'preferential formation of salt-active corticoids' thesis is hardly acceptable until such time as metabolic changes compatible with the elaboration of excess quantities of salt-active hormones are demonstrated in patients with collagen disease.

A number of theories attempting to explain the therapeutic action of ACTH

and cortisone have already appeared in the literature. Included among these are the following: *1*) interference with the release or the toxic action of the anaphylactogenic substance of the antigen-antibody reaction (96); *2*) alterations in cell permeability through action on hyaluronidase (329, 410); and *3*) suppression of mesenchymal tissue, in particular, inhibition of the development of granulation tissue (366). It is too early to evaluate critically the relative merits of these theories.

It would appear that the therapeutic action of ACTH and cortisone in the collagen diseases is pharmacological rather then physiological in nature. If the effect were physiological then the hormones would be expected to replace a deficiency. However, patients with collagen disease do not have a metabolic derangement characteristic of adrenocortical insufficiency. Furthermore, the ACTH-eosinophil test indicates that the available reserve of adrenocortical secretory activity is not noticeably impaired in the rheumatic state (465). Additional evidence in support of a pharmacological action is the fact that therapeutic doses of ACTH and cortisone usually produce some degree of hypercorticism.

Another view has been presented (393). In the collagen diseases certain tissues, e.g., connective tissue, may be relatively insensitive to cortical hormone; therapeutic maintenance of eucorticism in such 'deficient' connective tissue could be accomplished only at the price of inducing hypercorticism in cells with normal sensitivity to cortical hormone. If this concept is correct, then the defect in the pituitary-adrenocortical system is not in the pituitary or in the adrenal but in special peripheral tissues. This is an intriguing possibility with heuristic and practical implications.

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THE 17-KETOSTEROIDS: THEIR ORIGIN, DETERMINATION AND SIGNIFICANCE

HAROLD L. MASON AND WILLIAM W. ENGSTROM

From the Department of Biochemistry, Mayo Foundation

ROCHESTER, MINNESOTA

and from the Department of Internal Medicine, Yale University School of Medicine

NEW HAVEN, CONNECTICUT

IN 1931 BUTENANDT (20) described the isolation from urine of males of a crystalline steroid which in 1934 was identified as androsterone (22), the first of the class of substances now known as 17-ketosteroids. A number of compounds belonging to this group have since been isolated and methods have been developed for their quantitative determination. The qualitative pattern of the urinary 17-ketosteroids may be important for an understanding of certain diseases, and measurement of their quantity is often of aid in clinical diagnosis and in the interpretation of changes in testicular and adrenal cortical function. It is the purpose of this review to consider only those urinary steroids with a ketone group at carbon-17 of the steroid nucleus and such steroids as may be related to them as precursors or metabolites. Another fraction of urine contains steroids with biologic actions similar to the native adrenal steroids. This fraction is sometimes termed the 'corticosteroid' fraction. The chemical nature of this fraction is not clear, but probably it is composed largely of steroids with a sidechain at C-17. It will be discussed only as it may be related to the 17-ketosteroids.

NATURE AND ORIGIN

Nomenclature. The generic term 'steroids' was suggested by Callow and Young (34) for the group of biologically important compounds related to the familiar plant and animal sterols. The steroids include the bile acids, cardiac aglycones, sapogenins, sex hormones, adrenal cortical hormones and various derivatives of these substances. They all have in common a 4-ring nucleus consisting of a cyclopentane ring attached to a hydrogenated-phenanthrene ring system and named 'cyclopentanoperhydrophenanthrene.' In addition most steroids have methyl groups placed as shown in figure 1. The positions of these methyl groups ordinarily are indicated by a short line. The figure also shows the system used for numbering the carbon atoms of the nucleus.

A steroid compound may be identified by several different names. Throughout this review the common, or trivial, names will be used when possible rather than the systematic names which are usually more cumbersome. In figure 1 are shown the formulas of 6 commonly occurring urinary 17-ketosteroids, their ordinary names and systematic names.

In order to emphasize relationships to the simpler 17-ketosteroids such terms as 11-hydroxyandrosterone (for androstane-3(α),11(β)-diol-17-one), 11-hydroxyetio-

cholanolone (for etiocholane-3(α),11(β)-diol-17-one) and 11-ketoetiocholanolone (for etiocholan-3(α)-ol-11,17-dione) have been used. These names may be justified for the purpose of emphasizing the presence of the 11-oxygen atom, although 'hydroxy-' has the same meaning as '-ol-' and 'keto-' the same meaning as 'one.'

Hydroxyl groups at C-3 and C-11 (and other positions as well) may be oriented in two different positions with respect to the rest of the molecule. In the usual projection formula the orientation is designated by a solid bond linking the hydroxyl group with the carbon atom in question to indicate that the hydroxyl group lies

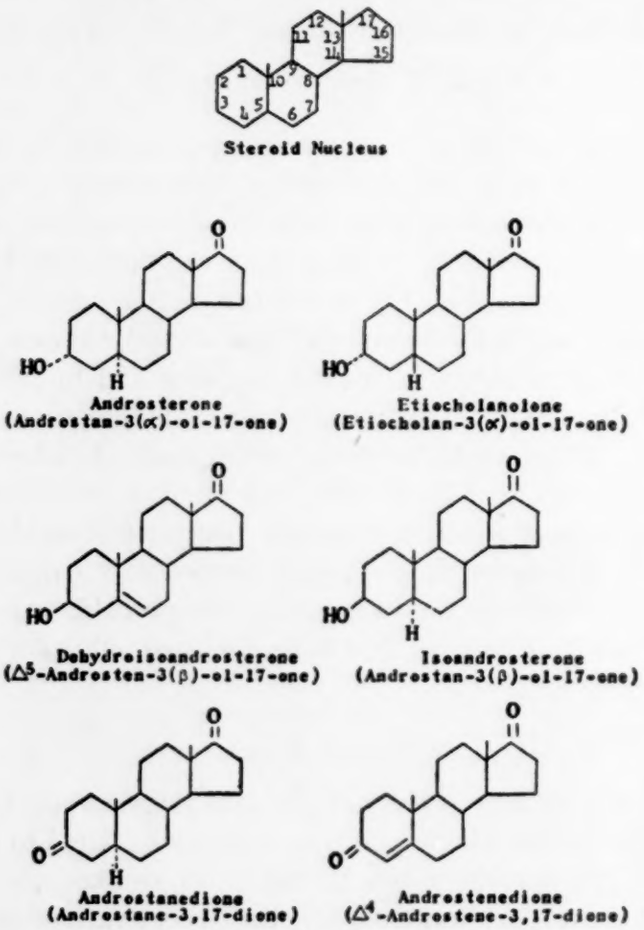

FIG. 1

above the plane of the molecule and a broken bond to indicate that the hydroxyl group lies below the plane of the molecule. These orientations are designated in the name by (β) and (α), respectively. Isoandrosterone and dehydroisoandrosterone are 17-ketosteroids which have 3(β)-hydroxyl groups and probably constitute the major portion of the so-called β-fraction. The significance of this fraction is discussed later.

Detailed discussions of nomenclature pertaining to steroids can be found in the article by Mason (165) and in the book by Fieser and Fieser (83).

Nature of the Neutral 17-Ketosteroids. The 17-ketosteroids comprise a group of steroid compounds which, as the name indicates, have in common a ketone group at position 17 of the steroid nucleus. They may differ in other structural features but

because of the presence of the 17-keto group they are characterized by certain color reactions which are discussed later. Some of the urinary 17-ketosteroids have androgenic properties but others such as etiocholanolone are biologically inactive. Estrone is also a 17-ketosteroid, but since it has a phenolic ring, it is acidic in nature and is removed from urinary extracts when they are washed with alkali. Therefore, it is not included among the neutral 17-ketosteroids. Throughout this review the term '17-ketosteroids' will be used to designate only the neutral fraction.

The 17-ketosteroids that have been isolated by extraction of urine are listed in table 1; included also in table 1 are brief comments on the known biologic properties, relation to disease, and known or probable precursors. The formulas of most of them are shown in figures 1 and 2. Under normal circumstances both androsterone and etiocholanolone are found in much larger amounts than any of the other urinary 17-ketosteroids. Thus, the major portion of the excreted neutral steroids have an *alpha* configuration of the hydroxyl group at carbon-3 and do not contain an oxygen function at carbon-11. Furthermore, it will be noted that some of the isolated steroids are transformation products which arise during processing of the urine. The significance of these artifacts is the same as that of the parent substance. Before hydrolysis all of these compounds are undoubtedly present in urine as conjugates of sulfuric or glucuronic acids, or of other unidentified substances.

Glandular Source. When testosterone (fig. 3) or its propionate is given to human subjects there is an increased excretion of 17-ketosteroids. Androsterone (25, 70, 72, 222), isoandrosterone (68, 71) and etiocholanolone (25, 67, 222) have been identified as metabolites of testosterone which are excreted in the urine. Since testosterone probably is produced by the human testes, it is reasonable to conclude that part of the urinary 17-ketosteroids results from testicular function. This conclusion is supported by observations that in some instances impairment of testicular androgen production or castration results in a decreased excretion of 17-ketosteroids. Further support is afforded by the case of interstitial cell tumor of the testis reported by Venning *et al.* (258). In this case 1015 mg., on the average, of 17-ketosteroids was excreted in the urine in 24 hours. Also, Landau and Knowlton (147) observed an increase in the excretion of 17-ketosteroids when rather large amounts of chorionic gonadotrophin were administered to normal men. Such an effect was not produced in a eunuchoid patient. Stimulation of the normal testes to produce more androgens was indicated by a pronounced retention of nitrogen, phosphorus and administered creatine.

Normal men excrete, on an average, more 17-ketosteroids than do normal women. Men suffering from destruction of the adrenal cortex, as in Addison's disease, may excrete several milligrams of 17-ketosteroids per day whereas many women excrete negligible amounts under the same circumstances. This observation is pertinent when it is considered that the adrenal cortex is the chief source of the precursors of the urinary 17-ketosteroids as will be shown presently. Thus, there is good evidence of a testicular contribution to the urinary 17-ketosteroids. There may be testicular steroids, other than testosterone, which give rise to the 17-ketosteroids, but if so, they remain unknown.

Inasmuch as orchectomy by no means abolishes the excretion of 17-ketosteroids,

TABLE 1. NEUTRAL 17-KETOSTEROIDS WHICH HAVE BEEN ISOLATED FROM NORMAL AND PATHOLOGIC HUMAN URINES (C-19 STEROIDS)

1. ANDROSTERONE. (*Androstan-3(α)-ol-17-one*). Possesses 1/10 the androgenic activity of testosterone. Isolated from urine of normal (20) and castrate men (28), normal (26) and castrate women (125), girl (275) and women (167) with adrenal tumor, pregnant women (161), girls with adrenal hyperplasia (167) and a man with an interstitial cell testicular tumor (258). A metabolite of testosterone and *3*.[1] Source of precursor: adrenal cortex and testes.

2. ETIOCHOLANOLONE. (*Etiocholan-3(α)-ol-17-one*). No androgenic activity. Isolated from urine of normal and castrate men (28), normal women (27), girls and women with adrenal tumor (167) and hyperplasia (167). A metabolite of testosterone and *3*. Source of precursor: adrenal cortex and testes.

3. DEHYDROISOANDROSTERONE. (Δ^5-*Androsten-3(β)-ol-17-one*). Possesses 1/3 the androgenic activity of androsterone. Isolated from urine of castrate men (28), normal (26) or castrate women (126). Marked excess usually excreted in cases of adrenal tumor (43) and slight excess in adrenal hyperplasia (43). Has never been demonstrated to be a metabolite of testosterone. Source undoubtedly the adrenal cortex alone but unknown whether it arises as such, or from some precursor.

4. ISOANDROSTERONE. (*Androstan-3(β)-ol-17-one*). A weak androgen. Isolated in small amounts from urine of normal women (194) and women with adrenal hyperplasia (23). A minor metabolite of testosterone in man (68). Isolated infrequently, and then in small amounts.

5. ANDROSTANEDIONE. (*Androstane-3,17-dione*). Isolated from urine of normal men and women (153). Probable precursors: testosterone and *3*.

6. ETIOCHOLANEDIONE. (*Etiocholane-3,17-dione*). Isolated from urine of normal men and women, women with hypertension and one woman with Cushing's disease (153).

7. ANDROSTENEDIONE. (Δ^4-*Androstene-3,17-dione*). Isolated from urine of a man with adrenal hyperplasia (153). Probable precursors: testosterone or *3*.

8. 11-HYDROXYANDROSTERONE. (*Androstane-3(α),11(β)-diol-17-one*). Possesses 1/4 the androgenic activity of androsterone (174). Isolated from urine of girls and women with adrenal tumor or hyperplasia (162, 167) and from urine of normal men (163); also, from girls with 'virilism' (174). Likely source of precursor: adrenal cortex.

9. 11-HYDROXYETIOCHOLANOLONE. (*Etiocholane-3(α),11(β)-diol-17-one*). Source of precursor: adrenal cortex (154). This compound is the precursor of *17*.

10. 11-KETOANDROSTERONE. (*Androstane-3(α)-ol-11,17-dione*). Source of precursor: adrenal cortex (154).

11. 11-KETOETIOCHOLANOLONE. (*Etiocholan-3(α)-ol-11,17-dione*). Isolated from urine of normal men and women and of certain patients with breast and prostatic carcinoma, lymphatic leukemia, 'adrenogenital' and Cushing's syndromes (153). Probably a normal metabolic product of one or more of the adrenal steroids.

12. Δ$^{2\ (or\ 3)}$-ANDROSTEN-17-ONE. Isolated from urine of ovariectomized women (126) normal men (60) and women with adrenal hyperplasia (60). A dehydration product of *1* (258).

13. Δ^{3,5}-ANDROSTADIEN-17-ONE. A weak androgen. Isolated from urine of 'normals' (60), ovariectomized women (125), man (19) and girl (275) with adrenal tumor, and women with adrenal hyperplasia (60). Undoubtedly a dehydration product of *3*.

14. 3-CHLORO-Δ^5-ANDROSTEN-17-ONE. Isolated from various urines by many investigators (26). Arises during hydrolysis of conjugates of *3* with hydrochloric acid.

15. Δ$^{9:11}$-ANDROSTEN-3(α)-OL-17-ONE. Isolated from urine of normal men and a girl with 'virilism' (153).

TABLE 1—*Continued*

16. Δ^{11}-ANDROSTEN-3(α)-OL-17-ONE. Possesses ¼ the androgenic activity of androsterone (73). Isolated from urine of girl with adrenal tumor (275) and girl with 'virilism' (73). Dehydration product of *8*. Apparently both *15* and *16* have been obtained, presumably by dehydration of *8*.

17. $\Delta^{9:11}$-ETIOCHOLEN-3(α)-OL-17-ONE. Dehydration product of *9*. Isolated (153) from urine of "a patient with breast carcinoma, certain patients with prostatic carcinoma, gastric carcinoma, lymphatic leukemia, essential hypertension and Cushing's syndrome"; also from 2 persons, 76 and 72 years of age, without known disease. Has not been detected in individual or pooled collections of urine from normal, younger men and women. Significance of this finding remains to be determined.

18. i-ANDROSTEN-6-OL-17-ONE.[2] Isolated from urine of a patient with an adrenal tumor.

[1] Numbers in italics refer to the numbers of the compounds listed in this table.

[2] Dingemanse, Huis in't Veld and Hartogh-Katz (55) have described recently the isolation of this 17-ketosteroid from the urine of a patient with an adrenal cortical tumor. It was identified by Barton and Klyne (8). Dingemanse *et al.* (56) concluded that this substance was excreted as such in an unconjugated form and that the dehydroisoandrosterone found in relatively large amounts in the urine of patients with adrenal cortical tumor must be regarded, to some extent at any rate, as an artifact produced by the action of boiling HCl on *i*-androsten-6-ol-one. This conclusion must be regarded with reservation in view of the procedure used for extraction. The urine was neutralized and refluxed for some time with successive portions of benzene. Since *i*-androsten-6-ol-17-one can be prepared by heating dehydroisoandrosterone *p*-toluene sulfonate with potassium acetate in aqueous acetone (21), it would be expected that dehydroisoandrosterone sulfate under these conditions also would yield the *i*-steroid. It is possible that the conditions used by Dingemanse *et al.* favor conversion of dehydroisoandrosterone sulfate, which presumably has been identified in urine (166, 179) to the *i*-compound. However, in view of the report of Dingemanse *et al.*, the identity of the supposed dehydroisoandrosterone sulfate isolated from urine requires further substantiation.

and since women excrete almost as much as men, it is necessary to look for a source other than the testes. Hirschmann (125, 126) was able to isolate from the urine of ovariectomized women the same 17-ketosteroids and in essentially the same quantities as those isolated from the urine of normal women by Callow and Callow (27). Although there is some indirect evidence that the ovaries normally may make a slight contribution to the urinary 17-ketosteroids, the amount must be very small. In view of the uncertainties involved, the ovary will not be considered further in this connection.

The only other known source of steroid hormones is the adrenal cortex. When the cortex is destroyed there is a marked reduction in the amount of urinary 17-ketosteroids. Indeed, in women these compounds frequently almost disappear from the urine. The small amount that may be found possibly reflects the nonspecificity of the reactions used for measurement of the 17-ketosteroids but there may be a small residue of functioning cortex in some patients. On the other hand, adrenocortical tumors usually are associated with large amounts of urinary 17-ketosteroids as well as other steroids. The adrenal cortex has been implicated further by isolation from normal urine of 17-ketosteroids that are oxygenated at position 11 of the steroid nucleus (figs. 1 and 2). Only the adrenal cortex is known to produce steroids oxygenated in this position.

To summarize, the urinary 17-ketosteroids are products of the adrenal cortex in women and of the adrenal cortex and testes in men. Variations in the amount excreted, especially in certain pathologic conditions, are in some measure reflections

of variations in the amounts of steroids produced by these two glands. It would be expected that any condition which affects the function of these glands would result in a corresponding change in the amount of urinary 17-ketosteroids. However, it should be pointed out that factors which may influence the rate or extent of transformation of precursor hormone to excretory product are not understood.

Chemical Precursors of 17-Ketosteroids and Transformations of Steroids in Relation to the 17-Ketosteroids. While testosterone (fig. 3) is undoubtedly a precursor of 17-ketosteroids, the nature of the adrenal cortical precursors is largely unknown. The cortical hormones that have been isolated in crystalline form have a two-carbon

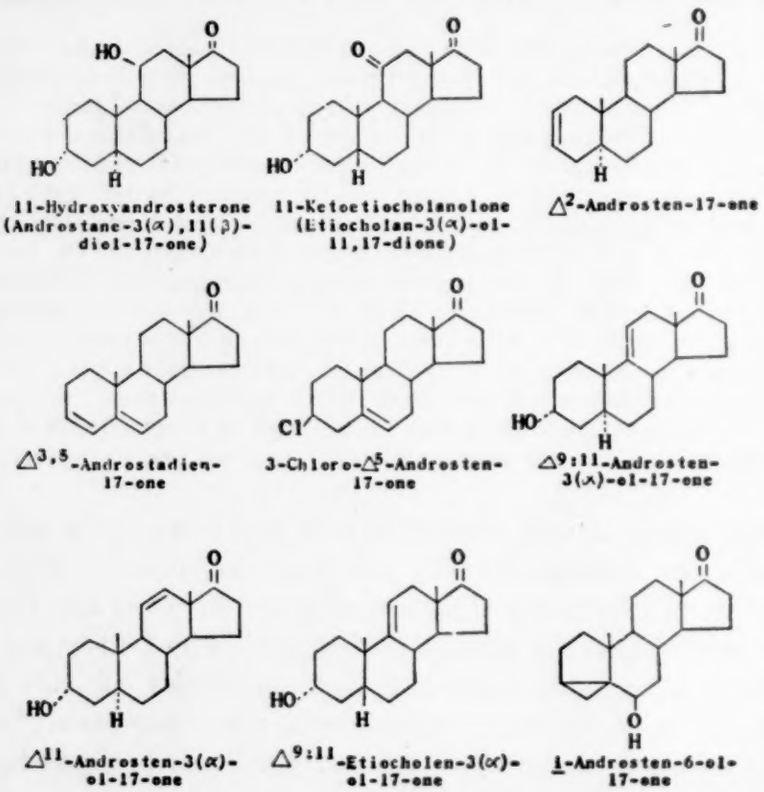

FIG. 2

side chain at C-17 of the steroid nucleus. It would be necessary to remove this side chain in order to convert one of them to a 17-ketosteroid. It appears that this conversion is not readily accomplished. It has not been possible to affect the excretion of 17-ketosteroids by administration of desoxycorticosterone (45) or of 11-dehydrocorticosterone (231) (fig. 3). The former is converted in small part to pregnanediol (84, 133) and the latter to 11-ketopregnanediol (164). However, administration of a large amount of 17-hydroxy-11-dehydrocorticosterone (cortisone) did produce a small increase in the excretion of 17-ketosteroids in patients with Addison's disease (232, 233). The possible relation between 17-hydroxy-11-dehydrocorticosterone and 11-hydroxyandrosterone, one of the urinary 17-ketosteroids, is illustrated in figure 3. However, the source of the major 17-ketosteroids, for example, androsterone or etiocholanolone, which do not have an oxygen atom at C-11, still remains unknown. Von Euw and Reichstein (82) have isolated from adrenal extracts Δ^4-androstene-3, 17-

dione (fig. 2), a compound which is known to serve as a precursor of androsterone and etiocholanolone, but the amount isolated was small and the possibility exists that it was formed by oxidation during the working up of the extract (209). Another adrenal steroid, 17-hydroxyprogesterone (199) (fig. 3) which itself is androgenic, may be a further source of 17-ketosteroids by metabolic removal of the side chain. Such removal appears to be facilitated by the presence of a hydroxyl group at C-17.

According to present knowledge, dehydroisoandrosterone (28) (fig. 1) which was mentioned as one of the two known members of the β-fraction, is a product of the adrenal cortex only. Hence an increase in the amount of this substance excreted,

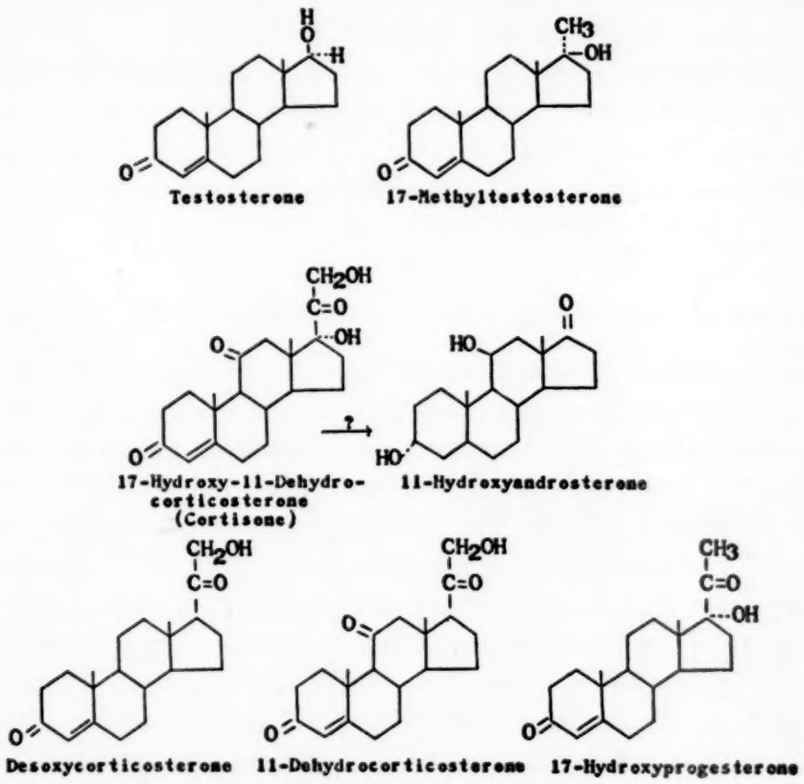

FIG. 3

and consequently an increase of the β-fraction, should be indicative of increased adrenal cortical activity. However, it will become evident that a quantitative measure of the β-fraction cannot invariably be relied on to give an accurate general index of adrenal cortical function.

EFFECT OF EXOGENOUS HORMONES

Steroid Hormones. Although administration of testosterone (fig. 3) or its propionate will increase the excretion of 17-ketosteroids, 17-methyl testosterone (fig. 3) and certain other 17-methyl steroids do not increase, and when given in sufficient quantities may decrease, the excretion of 17-ketosteroids (50, 88). Presumably the 17-methyl group cannot be removed readily with production of a 17-ketone group. The decreased excretion of 17-ketosteroids accompanying administration of 17-methyl testosterone was considered by Reifenstein *et al.* (211) to result from suppression of

production by the anterior pituitary of a trophic hormone which stimulates both gonads and adrenal cortices.

Hamblen *et al.* (114) observed decreases of 14 to 26 per cent in the excretion of 17-ketosteroids when estrogens were administered to 22 women with various grades of ovarian failure who excreted moderately elevated amounts of 17-ketosteroids before treatment. Estrogens would be expected to have much the same effect on pituitary function as methyl testosterone. Administration of estrogens has been shown to diminish the excretion of 17-ketosteroids by a patient with Cushing's syndrome (50) and by a female pseudohermaphrodite (217). Administration of progesterone does not cause a rise in urinary 17-ketosteroids, nor is there any evidence that it influences the amounts excreted (196).

Adrenocorticotrophic Hormone. The pituitary adrenocorticotrophic hormone (ACTH) stimulates increased excretion of 17-ketosteroids and of corticosteroids and the amounts may be large if sufficient ACTH is given (40, 86, 170, 248). The increased excretion of both types of steroids is maintained during administration of ACTH.

METABOLISM OF STEROIDS IN RELATION TO THE URINARY 17-KETOSTEROIDS

In Vivo Metabolism. Testosterone and the adrenal steroids have been discussed as precursors of the urinary 17-ketosteroids. Other steroids which may be intermediary metabolites have been administered to human subjects to test their effect on the excretion of 17-ketosteroids and to determine what products would appear in the urine. In addition to testosterone, androsterone, dehydroisoandrosterone (fig. 1), Δ^4-androstene-3,17-dione (fig. 2), androstane-3(α),17(α)-diol[1] (or its diacetate), androstane-3,17-dione (fig. 2) and Δ^5-androstene-3(β),17(α)-diol[1] cause an increase in the androgenic activity and 17-ketosteroid content of urine (72, 88). It should be noted that all of these steroids contain 19 carbon atoms; none contain the 2-carbon side chain at C-17. Dorfman and Hamilton (72) isolated androsterone from the urine when they administered the first 6 of these compounds by mouth to men with organic and functional testicular deficiency. Only dehydroisoandrosterone failed to yield androsterone. The relative inability of the body to metabolize androsterone further was indicated by isolation of 24 per cent of the androsterone administered.

Mason and Kepler (168, 169) found that when dehydroisoandrosterone acetate was injected intramuscularly into human subjects not only was there an increase in the excretion of 17-ketosteroids, but also androsterone and etiocholanolone could be isolated from the urine in relatively large amounts. In some cases a small part of the dehydroisoandrosterone was recovered unchanged.

Miller and Dorfman (173) isolated dehydroisoandrosterone from the urine of male guinea pigs after they were given injections of Δ^5-androstene-3(β),17(α)-diol[1] (as the acetate), a substance which was isolated from urine of human beings as a metabolite of dehydroisoandrosterone (168). This metabolite also was the chief prod-

[1] The assignment of the (α) configuration at C-17 is taken from the paper as published. The configuration is now known to be 17(β) (83).

uct isolated by Schneider and Mason (223) after incubation of dehydroisoandrosterone with slices of rabbit liver. Although different species were involved, the reversible relation of these two steroids is indicated. This reversible relation was also demonstrated in studies conducted *in vitro* with liver and kidney tissue.

In Vitro Metabolism. The site of transformation of the precursors to the urinary 17-ketosteroids is not known with certainty although there is considerable evidence which implicates the liver. Samuels, McCaulay and Sellers (220) found that incubation of testosterone with minced liver from rats resulted in disappearance of the 4-ethylenic-3-ketonic group. There was no evidence of formation of 17-ketosteroids. The reaction involved in the destruction of testosterone appeared to be an oxidative enzymic one involving metals, sulfydryl-containing enzymes, and the Krebs cycle. In a later study Sweat and Samuels (236) showed that the addition of diphosphopyridine nucleotide, citrate and nicotinamide increased the rate of destruction of testosterone by rat livers. Diphosphopyridine nucleotide caused large amounts of 17-ketosteroids to appear while citrate did not.

On the other hand, Clark and Kochakian (37) found that without any additives minced rabbit liver converted testosterone to Δ^4-androstene-3,17-dione, epitestosterone (cis-testosterone) and small amounts of unidentified steroids. More than half of the testosterone was recovered unchanged. The usual urinary products of testosterone metabolism, that is, androsterone and etiocholanolone, were not found. Clark, Kochakian and Lobatsky (38) then showed that Δ^4-androstene-3,17-dione could be converted by rabbit liver to testosterone and epitestosterone. It will be noted that the changes which could be identified all occurred at C-17 and that these were the predominant reactions (fig. 4).

Schneider and Mason (224) found that when androsterone and etiocholanolone were incubated with slices of rabbit liver small amounts of androstanedione and etiocholanedione, respectively, could be isolated, showing that oxidation at C-3 had occurred. Also, isolation of isoandrosterone, a 3(β)-hydroxy steroid, after incubation of androsterone, a 3(α)-hydroxy steroid, showed that the oxidation at C-3 was reversible since inversion of the orientation of the 3-hydroxyl group must have involved oxidation to a 3-ketone group followed by reduction to a hydroxyl group with the β-configuration (fig. 4). Conversion of dehydroisoandrosterone to androsterone or etiocholanolone by rabbit liver could not be demonstrated (223). Thus, it has been shown that the liver *in vitro* is capable of converting 17-hydroxy compounds to 17-keto compounds and vice versa, but under these circumstances the equilibrium greatly favors the 17-hydroxy compounds.

Kochakian and his associates (145) found that homogenates of kidneys of rabbits and guinea pigs converted testosterone in part to Δ^4-androstene-3,17-dione accompanied by small amounts of unidentified ketosteroids. Homogenates of liver as well as kidney converted less than 5 per cent of androstene-3(α),17(α)-diol to one or more ketosteroids. It appears that kidney is not as active as liver toward these steroids but that the metabolic reactions are essentially the same. Thus far, products which would result from reduction of a Δ^4 or Δ^5 double bond have not been isolated after incubation of compounds containing such bonds with liver or kidney preparations.

METHODS FOR DETERMINATION OF URINARY 17-KETOSTEROIDS

Collection and Preservation of Urine. Urine may be collected in glass bottles which contain toluene, 5 to 10 ml. of concentrated hydrochloric acid, (202, 249), or 10 ml. of glacial acetic acid as preservatives. The acidified urine may be stored in a refrigerator for several days and probably longer without loss of 17-ketosteroids. Usually an aliquot of 0.2 to 0.5 of the 24-hour specimen should be adequate for a determination, and methods have been devised for use of only a few milliliters (74, 175).

Hydrolysis of Conjugates and Extraction of Steroids. Hydrolysis of the conjugates of steroids presents a problem which has not been satisfactorily solved. Present methods of hydrolysis with inorganic acids may destroy steroids and it is known that in some cases they do cause alteration of the molecule (205). Further, the individual conjugates may differ in their stability. Some authors (9, 244, 251) have

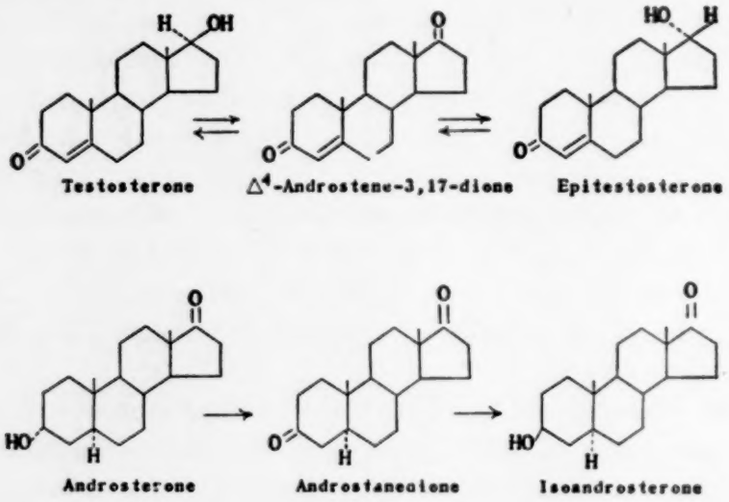

FIG. 4

recommended simultaneous hydrolysis and extraction to avoid production of dehydration products through loss of the 3-hydroxyl group of so ne of the 17-ketosteroids. It has become evident, however, that such procedures do not entirely prevent the loss of the 3-hydroxyl group from some of the 17-ketosteroids present and that with solvents such as carbon tetrachloride or benzene the temperature of the mixture may not allow complete hydrolysis (94).

There is no general agreement on the conditions of hydrolysis which lead to optimal results. Ideally, hydrolysis should quantitatively free the neutral steroids from their conjugated state and should do so without destruction or alteration of their molecular structure. Most often the urine is boiled for 10 minutes after addition of 0.1 of its volume of concentrated hydrochloric acid. It is then cooled quickly and extracted. Some workers prefer to use an equivalent or even greater amount of sulfuric acid (63, 193). Others (132) increase the amount of hydrochloric acid to 0.15 of the volume of urine. The boiling time may be reduced to 7 minutes (202, 217) or extended to 15 minutes (235). Autoclaving of acidified urine at 15 pounds pressure for 15 minutes, as described by Cuyler and Baptist (47), or prolonged boiling (198), tends to destroy some of the 17-ketosteroids (31, 178).

Talbot, Ryan and Wolfe (245) introduced a novel method of hydrolysis in order to prevent conversion of 3(β)-hydroxy-17-ketosteroids to nonalcoholic ketosteroids. The method involved extraction of urine with butyl alcohol at pH 7.0, solution of the extract in sodium acetate buffer, pH 5.8, addition of barium chloride and heating the mixture in a boiling water bath for 4 hours. This procedure works well with dehydroisoandrosterone sulfate but the sulfates of other 17-ketosteroids, such as androsterone and particularly the glucuronides of steroids, are not hydrolyzed readily by this procedure.

Bitman and Cohen (14) found that only 43 to 68 (average 58) per cent of the 17-ketosteroids present in urine were extracted by butyl alcohol at pH 7.0 although quantitative extraction was achieved at pH less than 3.0. However, an average of only 40 per cent of the 17-ketosteroids extracted by butyl alcohol at pH 7 was liberated from conjugation by heating with barium chloride. The remainder could be recovered after hydrolysis with HCl. In the fraction released from conjugation by the barium chloride hydrolysis the β-fraction was approximately 25 per cent of the total 17-ketosteroids in the butyl alcohol extract and represented an average of 13.38 per cent of the total 17-ketosteroid content of urine, whereas after acid hydrolysis of urine the β-fraction averaged only 4.5 per cent of the total 17-ketosteroids. It is evident that the β-fraction may comprise a greater percentage of the total 17-ketosteroids than has been previously indicated from data derived from procedures involving hydrolysis with hydrochloric acid.

Extraction of the free 17-ketosteroids after hydrolysis of the conjugates may be accomplished by shaking in a separatory funnel with an organic solvent or by use of a continuous extractor. The latter procedure is very convenient when many determinations have to be made.

Carbon tetrachloride, benzene and ether have been used widely for extraction. Ethyl ether extracts more of the urinary pigments than does carbon tetrachloride or benzene. Butyl ether extracts little of the urinary pigments (44, 155) but it has the disadvantage of a relatively high boiling point. Ethyl and butyl ethers must be purified to remove chromogenic material (31, 238).

The urinary extract is washed with a solution of base in order to remove acids, phenols and some color. Talbot and associates (244) introduced sodium hydrosulfite ($Na_2S_2O_4$) into the sodium hydroxide wash. This step results in bleaching of the reddish-brown extract to a pale yellow color and appears to have value in that much undesirable coloring matter is removed.

Most investigators (24, 90, 149, 205, 243) have found that if charcoal is used to remove color from ether extracts, variable amounts of 17-ketosteroids are lost on the charcoal. However, Benda and Bixby (11) claim that treatment of a carbon tetrachloride extract with 2 gm. of potassium carbonate and 0.2 gm. of Norit A charcoal does not cause a significant loss of 17-ketosteroids.

After these procedures the neutral extract is ready for analysis. It may be assayed directly for total 17-ketosteroid content by proper application of colorimetric technics. However, its chemical composition may be more fully characterized by fractionation procedures (202). The extract may be treated with digitonin to separate the 3(β)-hydroxy steroids. Under some circumstances it may be desirable to employ Girard's reagent *T* or *P* (101) to separate the ketonic fraction, containing the 17-

ketosteroids, from the nonketonic fraction. The alcoholic 17-ketosteroids may be separated from the nonalcoholic fraction with the aid of succinic anhydride. Individual steroids may be separated by chromatography. The details of the more important phases of these procedures are discussed in the following sections.

Zimmermann Reaction. The colorimetric methods that have been used most widely for the determination of 17-ketosteroids are based on the reaction of the group —CH_2CO— with *m*-dinitrobenzene in alkaline solution. Zimmermann (280, 281) demonstrated the usefulness of this reaction for the quantitative determination of pure ketonic steroids. Hence the reaction is usually known as the Zimmermann reaction when it is applied to steroids although von Bittó (15) had previously applied it to other ketones. Steroids with a ketone group at C-17 yield a red color with an absorption maximum at 520 mμ (90, 132, 224). Ketone groups at other positions of the steroid nucleus or in a side chain also may yield colored products. However, under the conditions of assay described by Callow *et al.* (30) and Holtorff and Koch (132), these ketones may be ignored in so far as the ordinary neutral urinary extract is concerned (117). However, in some special cases, such as pregnancy, in which relatively large amounts of other ketones may be excreted, the effect of these other ketones may not be ignored (256).

Wu and Chou (279), Oesting (188, 189) and Neustadt (187) applied the method described by Zimmermann to urinary extracts. Wu and Chou used absolute alcohol as the solvent for all reagents while Oesting and also Neustadt used aqueous alcohol in the reaction mixture. Friedgood and Whidden (95–97) and Strickler *et al.* (235) have made critical studies of these latter methods. They have been discarded as more sensitive and accurate methods were developed.

The method of Wu and Chou (279) was developed further by Callow *et al.* (30). Since their procedure, with some modifications, has been widely adopted, its essential details are outlined. To 0.2 ml. of test solution in absolute alcohol is added 0.2 ml. of a 2 per cent solution of *m*-dinitrobenzene and 0.2 ml. of 2.5 ± 0.02 N potassium hydroxide, both in absolute alcohol. The color is allowed to develop at 25 ± 0.1° C. for one hour with the solution shielded from all but diffused light. After dilution with 10 ml. of absolute alcohol the intensity of the color is determined. A blank is prepared in the same manner except for substitution of absolute alcohol for the test solution.

The variable factors which influence the color reaction have been studied by many investigators, including the purity and the concentrations of the reagents, the relative amounts of the reactants, the amount of water in the alcohol solutions and the duration of color development. Some of these factors are considered in detail.

Callow, Callow and Emmens emphasized the importance of purification of the *m*-dinitrobenzene and absolute alcohol and described methods for their purification.

The use of the 2.5 N alcoholic potassium hydroxide presents a problem since the alcoholic solution deteriorates after a short time and a yellow color develops even when it is stored at a low temperature. However, Wilson and Carter (272) added ascorbic acid to the KOH and found that it would then keep for at least three months if stored under nitrogen. Although the concentration of KOH must be adjusted accurately in order to duplicate results, the concentration may be allowed

to vary somewhat more than the limits specified by Callow *et al.*, if a standard is run with each set of unknowns. It may be commented here that the amount of color developed with a given amount of 17-ketosteroids changes gradually as the reagents age. It is, therefore, likely that the use of a standard calibration curve may lead to rather large errors, and it is highly desirable that a standard be included in any series of determinations. Wilson and Carter found that their results were more reproducible when the KOH was stabilized with ascorbic acid.

The instability of alcoholic KOH has led some investigators to use aqueous KOH. However, it is necessary to use a more concentrated aqueous solution to produce maximal color. Holtorff and Koch (132), whose procedure is widely used, found that 5 N potassium hydroxide was satisfactory. Also, they substituted 95 per cent alcohol for absolute alcohol in all other solutions. In addition to the usual reagent blank, they introduced a blank of 0.2 ml. of urinary extract, 0.2 ml. of 95 per cent alcohol and 0.2 ml. of the 5 N aqueous KOH This blank was used to compensate for the colored impurities present in the extract. Carbonate which may be present in the aqueous KOH precipitates on dilution of the reaction mixture with 95 per cent alcohol after development of the color. However, 60 to 75 per cent alcohol may be used for dilution without precipitation of carbonate (205, 214). The optimal time for development of color depends on the method used. Nathanson and Wilson (185) found that when the technique of Callow *et al.* was applied to urinary extracts, 60 to 80 minutes were required for development of maximal color, while with the techniques in which aqueous alkali was used, at least 105 minutes were necessary, although Holtorff and Koch used only 45 minutes. Hansen *et al.* (117) chose 60 minutes as an optimal time for a procedure essentially that of Holtorff and Koch. These discrepancies suggest the desirability for each user of a method to determine the optimal time for development of color by urinary extracts and by the reference 17-ketosteroid under the conditions of the method used.

Wu and Chou (279) noted that between 20° and 30°C. the intensity of color increased approximately 3 per cent per degree of temperature rise. Callow *et al.* (30) observed that less color was developed when the reaction mixture was exposed to bright light and that the color faded slowly after dilution; fading was accelerated by exposure to bright light and air.

The detailed procedures proposed by Callow, Callow and Emmens and by Holtorff and Koch have received critical study and some modifications have been proposed. The value of a strongly alcoholic medium and of small volumes of fairly concentrated reagents became apparent. Friedgood, with Whidden (97) and Berman (93), found that dehydroisoandrosterone gave more color than androsterone when the reaction was carried out in aqueous alcohol. Holtorff and Koch, however, achieved an equivalence of chromogenicity by increasing the concentration of the aqueous KOH to 5 N. A more serious defect resulting from the presence of water was the failure of the crude urinary extract to produce an amount of color proportional to the amount of extract used. Engstrom and Mason (79), using the Holtorff-Koch method, and Strickler *et al.* (235), using the older Oesting method, demonstrated that the color developed by the urinary extracts did not strictly obey Beer's law. However, when pure 17-ketosteroids, or when purified ketonic fractions of urine ex-

tracts were used, the color produced by the Holtorff-Koch method was proportional to the amount of material used. On the other hand, the use of absolute alcohol for all reagents as in the method of Callow, Callow and Emmens obviated the deviation from proportionality exhibited by the crude urinary extract. Thus the evidence indicates that when the Zimmermann reaction is applied to crude urinary extracts, the techniques which use absolute alcohol as the reaction medium are likely to give more accurate results. Equally accurate results can be expected from the Holtorff-Koch procedure provided it is applied to the ketonic fraction of the crude extract.

The presence of chromogenic materials, other than 17-ketosteroids, contained in urine extracts constitutes another problem. These yield a brown color which absorbs appreciably at 520 mμ and a blank of extract and alkali without dinitrobenzene will not wholly compensate for its presence. If the concentration of 17-ketosteroids is low, the intensity of the brown color may be sufficient to interfere seriously with the determination. Three methods are available for eliminating in large part the effect of this interfering chromogenic material. Cahen and Salter (24) extracted the test solution with chloroform after the development of color and dilution with 60 per cent alcohol. The chloroform contained the red pigment which is characteristic of the 17-ketosteroids and left the brown color in the aqueous alcoholic phase. This procedure of Cahen and Salter appears to have merit, and it is a relatively simple method for eliminating the interfering pigments.

Another means of correcting for the presence of chromogenic material in crude extracts involves the chemical purification of the extract. It has been shown by Talbot *et al.* (244) that the interfering chromogens occur largely in the nonketonic fraction of urinary extracts and that the pigments derived from them absorb maximally at about 410 mμ. The nonketonic fraction can be separated from the ketonic fraction with the aid of Girard reagent. Colorimetric assay of the purified ketonic fraction then gives a more accurate estimate of the amount of 17-ketosteroids than does direct, uncorrected assay of the crude fraction.

The possibility of using a color correction equation for eliminating the errors caused by the interfering chromogens in the crude extracts was suggested by Fraser *et al.* (90) and studied by Talbot *et al.* The use of a correction equation has been criticized on statistical grounds by Pincus (200). However, Talbot *et al.* (238) and Engstrom and Mason (79) found good correlation between values determined with the aid of the correction equation and values obtained with the isolated ketonic fractions of the same extracts.

The development of the correction equation is predicated on the assumption that absorption of light by the brown interfering pigment and by the red pigment derived from the 17-ketosteroids follows Beer's law. Since the evidence indicates that the total color developed by these chromogens in the crude urinary extract may not follow Beer's law when aqueous alkali is employed, it would be expected that application of the correction equation would not be valid under these circumstances. Engstrom and Mason found that in some instances serious errors were introduced by use of the equation with the Holtorff-Koch method. At present it appears that the equation must be used only with techniques which employ absolute alcohol.

Which 17-ketosteroid should be used as a reference standard also presents a

problem since urinary extracts contain mixtures of 17-ketosteroids. Nathanson and Wilson (185, 273) showed that different 17-ketosteroids had slightly different chromogenic capacities. However, the possible errors that might be introduced by this factor are minimized when the Holtorff and Koch or the Callow procedures are followed. Dehydroisoandrosterone appears to be the most satisfactory, and androsterone the least satisfactory steroid to use as a reference.

Pincus Method. Pincus (201) found that 17-ketosteroids react with antimony trichloride in acetic acid solution to give a greenish-blue color which is proportional to the amount of 17-ketosteroids. This reaction can be applied to crude extracts of urine and appears to be rather specific. However, androstanediol, which is not a 17-ketosteroid, gives a weak reaction and dehydroisoandrosterone, unlike the saturated 17-ketosteroids and $\Delta^{2(\text{or } 3)}$-androsten-17-one gives only a faint yellowish-blue color with this reagent.

Polarographic Methods. Wolfe, Hershberg and Fieser (276) found that 17-ketosteroids could be determined with the polarograph if the ketone group was first condensed with Girard's reagent *T*. The saturated 3-ketosteroids, similarly processed, were polarographically indifferent. The derivatives of 20-ketosteroids with Girard's reagent gave a characteristic wave attributable to the 20-ketone group. A micro-analytical method for determination of dehydroisoandrosterone and other Δ^5-3-hydroxy-steroids with the polarograph was devised by Hershberg, Wolfe and Fieser (122). Werthessen and Baker (268) offered a refinement which permitted distinction of the 17-ketosteroids from the 20-ketosteroids.

Barnett, Henly and Morris (6) treated urinary extracts in aqueous dioxan solution with potassium permanganate to remove substances which interfere with establishment of a true baseline for polarography. The method elaborated is stated to measure only neutral 17-ketosteroids. These authors, with Warren (7), compared the results obtained by use of the Zimmermann-Callow colorimetric method with those obtained by use of their polarographic method (tables 2 and 3). The colorimetric method gave values somewhat higher than those obtained with the polarographic procedure and the standard deviation of the colorimetric values was greater.

METHODS OF FRACTIONATION

Dehydroisoandrosterone and the β-Fraction. Determination of the β-fraction of the 17-ketosteroids is important in the differential diagnosis of adrenal cortical tumors. Methods for determination of this fraction are based on precipitation of the 3(β)-hydroxy-17-ketosteroids as digitonides followed by estimation of the quantity of 17-ketosteroids not precipitated or by estimation of the quantity of 17-ketosteroids in the digitonide after decomposition of the precipitate with pyridine and ether (87, 202, 243). In any of the procedures involving precipitation with digitonin, $\Delta^{3,5}$-androstadien-17-one and chlorodehydroandrosterone—both of which may be derived from dehydroisoandrosterone during acid hydrolysis—are not precipitated, but remain in the α-fraction. Since the formation of these substances depends largely on the method used for hydrolysis, any interpretation of values for the β-fraction must take into consideration the method used for hydrolysis of conjugates.

Dirscherl and Zilliken (58) found that upon treatment of a small amount of

TABLE 2. 17-KETOSTEROIDS IN THE URINE OF NORMAL MEN

REFERENCE	METHOD[1]	NO. OF SUBJECTS	AGE, YR.	17-KETOSTEROIDS, MG. PER DAY		REFERENCE	METHOD[1]	NO. OF SUBJECTS	AGE, YR.	17-KETOSTEROIDS, MG. PER DAY	
				Range	Mean					Range	Mean
(7)	Polar.	8	20–22	12.8–21.0	16.8	(139)	D	53	17–34	9.0–30.0	18.0
	C	8	20–22	12.7–27.5	18.4			11	35–49	7.0–23.0	15.0
								18	50–75	4.0–16.0	9.0
(9)	C	11	17–30	10.5–19.2	14.1	(156)	HK	40	20–40	4.9–18.4	9.1
(31)	C	10		3.5–15.0	9.0	(157)	HK	19	20–60	10.0–23.2	16.2
		5	71–75	1.8– 4.8	3.4						
(79)	C (corr.)	14	20–40	6.0–20.8	13.6[2]	(193)		12		9.4–20.9	13.3
(85)	C	73		6.7–27.2	12.5	(215)	P	14	20–58	9.5–23.0	14.2
							CS	14	20–58	8.5–23.0	15.9
(90)	C	9	20–40	8.1–22.6	13.8	(217)	CS	24	19–30	13.1–27.1	19.8
	(corr.)	4	71–75	1.8– 4.8	3.4			2	31–35	13.8–16.6	
(116)	HK	8	18–47	10.0–21.0	14.9	(226)	C	10	20–40	11.6–17.5	14.3
		11[4]	20–29	5.9–15.5	10.8						
		9[4]	30–39	5.2–20.0	10.1						
		11[4]	40–49	4.8–14.1	7.7						
		10[4]	50–59	3.6–10.7	6.0						
		7[4]	60–69	3.8– 6.9	4.8						
		3[4]	70–75	2.1– 4.0	2.9						
(121)	C[3]	20	20–40	5.4–21.2	11.4	(242)	C (corr.)				13.8
						(251)	TO	9	18–41	9.2–20.2	16.7
						(259)	HK	14	20–51	10.0–25.0	16.6
							(corr.)	2	77–81	10.0–10.6	
						(278)	HK	16[4]	20–29	4.0–24.0	11.5
								18[4]	30–39	6.0–17.0	11.7
								10[4]	40–49	7.0–15.0	11.3
								5[4]	50–59	8.0–12.0	9.5

[1] C = Callow, Callow and Emmens (30) CS = Cahen and Salter (24) D = Drekter *et al.* (74) HK = Holtorff and Koch (132) P = Pincus (201) Polar. = Polarographic (6) TO = Tompsett and Oastler (249) Corr. = values calculated with use of correction equation.

[2] This value is slightly less than the one previously reported since another individual with a consistent excretion of only 6 mg. was added to the series.

[3] Determinations on ketonic fraction separated with Girard reagent *T*.

[4] These subjects were prisoners.

pure dehydroisoandrosterone with concentrated sulfuric acid followed by an equal volume of water, a blue-violet color was produced which was not given by androsterone, testosterone, androstenedione, estrone, estradiol, progesterone, corticosterone or cholesterol. Patterson (192) applied this reaction to urinary extracts as a test for the presence of an adrenal cortical tumor.

Since dehydroisoandrosterone often is greatly increased in amount and is the chief constituent of the β-fraction when an adrenal cortical tumor is present, the direct determination of this substance should afford a more convenient and definitive

TABLE 3. 17-KETOSTEROIDS IN THE URINE OF NORMAL WOMEN

REFERENCE	METHOD[1]	NO. OF SUBJECTS	AGE, YR.	17-KETOSTEROIDS MG. PER DAY		REFERENCE	METHOD[1]	NO. OF SUBJECTS	AGE, YR.	17-KETOSTEROIDS MG. PER DAY	
				Range	Mean					Range	Mean
(7)	Polar.	20	18–37	5.3–18.1	11.9	(156)	HK	18	20–40	1.5–9.7	4.2
	C	20	18–37	6.2–22.4	13.2	(193)		12		3.5–14.6	7.4
(31)	C			1.7–12.6	6.8	(215)	P	14	22–54		7.9
(79)	C (corr.)	18	20–35	4.8–17.0[2]	10.0		CS	14	22–54		8.0
(85)	C	65		3.8–16.9	8.2	(226)	C	5		5.6–15.5	10.1
(90)	C	14	20–40	5.1–14.2	9.0	(242)	C	9			9.1
(121)	C[3]	19	20–40	4.2–11.7	7.6	(259)	HK	14	21–45	6.0–18.0	11.6
							(corr.)	2	74–76	5.8–8.2	
(139)	D	20	17–64	3.0–16.0	9.3	(265)	C	5		5.4–19.6	12.2

[1] C = Callow, Callow and Emmens (30) CS = Cahen and Salter (24) D = Drekter *et al.* (74) HK = Holtorff and Koch (132) P = Pincus (201) Polar. = Polarographic (6) Corr. = values calculated with use of a correction equation.

[2] Three values have been added since the original publication with slight decrease in the lower level.

[3] Determinations on ketonic fraction separated with Girard reagent *T*.

test for the presence of a tumor than evaluation of the total β-fraction by some such method as digitonin precipitation. Munson, Jones, McCall and Gallagher (180) have developed a method for determination of dehydroisoandrosterone. Their method, utilizing a modification of the Pettenkofer reaction, depends on the development of a color when dehydroisoandrosterone (or its transformation artifacts, chlorodehydroandrosterone and androstadienone, or both), furfural, acetic acid and sulfuric acid are heated together at 67 ± 0.2°. While it may not be possible satisfactorily to apply this method directly to crude urinary extracts, its application to the ketonic fraction appears to be highly specific. Further, since the hydrolytic artifacts of dehydroisoandrosterone also react in this method, the difficulties that arise with acid hydrolysis may be largely obviated.

Girard Separation. Several quantitative methods have been described (202, 205,

244) for separation of the crude neutral extract of urine into nonketonic and ketonic fractions by means of Girard's reagent *T*. However, it is unlikely that the ketonic fraction is composed of pure 17-ketosteroids. Possibly some 3-ketosteroids or 20-ketosteroids, or even nonsteroid ketones normally are present.

Adsorption Techniques. In an attempt to purify the crude extract and separate the ketonic fraction, Baumann and Metzger (9) adsorbed the steroids from pentane solution on magnesium oxide and eluted them with ether. This procedure has been used by several workers but Baumann, Metzger and Sprinson (10) found that purification with Girard reagent yielded values 50 to 100 per cent above those obtained with the use of magnesium oxide. Clearly, considerable steroid was lost when the adsorption technique was employed.

In order to separate alcoholic from nonalcoholic 17-ketosteroids, Talbot, Wolfe, McLachlan and Berman (246) allowed a solution of the urinary extract in carbon tetrachloride to percolate through a column of activated alumina. The alumina retained the alcoholic steroids and permitted the nonalcoholic ketones to pass through.

Dingemanse, Huis in't Veld and deLaat (57) described the adsorption on alumina and fractional elution of 17-ketosteroids with colorimetric determination of the amount of each fraction. Three of the fractions were identified as dehydroisoandrosterone, androsterone, and etiocholanolone. Another fraction has recently been identified as *i*-androsten-6-ol-17-one. Identification of other fractions was doubtful. Lieberman and his associates (153) have used a similar technique on a much more elaborate scale. A large number of crystalline 17-ketosteroids (and other steroids) obtained from normal and abnormal urines have thus been identified and the relative amounts determined.

The dinitrophenylhydrazones of 17-ketosteroids (136) have been separated by chromatographic analysis. It may be that these derivatives will lend themselves to fractionation, identification and quantitation for purposes of investigation.

Systematic Analysis of Urine. Pincus (202) has published an excellent scheme for the systematic fractionation and determination of urinary steroids, including the 17-ketosteroids, estrogens and corticosteroids (cortin fraction). Methods are given for the determination of total 17-ketosteroids and of the α-fractions and β-fractions, and for assays of the ketonic and nonketonic fractions for various principles.

THE 17-KETOSTEROIDS OF NORMAL[2] INDIVIDUALS

Adults (20 to 40 Years of Age). Published values for the amounts of 17-ketosteroids excreted by healthy adults have been collected in tables 2 and 3, which tables do not include all published values. Groups of values which were obtained from less than 5 individuals have not been included, and some of the earlier figures have been omitted because it was considered that the techniques used did not give optimal results. Most of the values have been obtained from individuals 20 to 40 years of age. The following discussion is confined to this age group. The 17-ketosteroid excretion of normal persons of the older age group and of children is considered separately.

[2] It is recognized that 'normal' is not a satisfactory adjective but we have used it for want of a better simple term. Normal refers to individuals or values obtained from individuals without obvious evidence of disease and subject to the influences of ordinary life.

With one or two exceptions there is general agreement that the values for the 17-ketosteroids excreted by normal young adult men and women cover a wide range and that there is considerable overlapping in the values of the two sexes. However, there is considerable disagreement as to the lower and upper limits of this range and the mean values. The disagreements may be attributed in part to the limited number of individuals studied in many instances and in part to the different methods used. Even so, it is difficult to account for some of the large differences.

The majority of the mean values for men (excluding prisoners) obtained by some application of the Zimmermann reaction (table 2) lies between 12.5 and 16.7 mg/24 hours with a lower limit of normal at about 6.0 mg. and an upper limit at about 25.0 mg/24 hours. The relatively low mean value for men (9.0 mg.) of Callow *et al.* (31) may perhaps be attributed in part to incomplete hydrolysis of conjugates in the presence of the relatively low concentration of acid used by them, although their careful comparison with other procedures makes this explanation doubtful. In the case of McCullagh *et al.* (156) who give a similar mean value, the ketonic fraction was separated with the aid of Girard reagent or with magnesium oxide according to Baumann and Metzger (9). Henriques and Henriques (121) also used only the ketonic fraction separated by means of Girard reagent and their mean value (11.4 mg.) is somewhat lower than the mean values obtained by the other workers who used the Callow modification of the Zimmermann reaction, with or without use of the correction equation. Henriques and Henriques showed that there was no appreciable loss of steroids in their application of the Girard fractionation. The similar low mean value (11.5–11.7 mg.) found for the same age group by Wooster (278) may be explained by the fact that all of these subjects were prisoners. Hamilton and Hamilton (116) found that the male prisoners whom they studied excreted on the average 4 mg/24 hours less than a comparable group of men who were not prisoners.

The extremely high mean value of 19.8 mg. given by Salter *et al.* (217) in 1948 is difficult to reconcile with other values unless it was due to the fact that none of these subjects was more than 30 years of age. The method used should give somewhat lower values than some of the other methods. However, Dorfman (69) has given even higher values which were obtained by use of simultaneous hydrolysis and extraction and the Holtorff-Koch method of assay; the range was 15.9 to 34.0 mg. per day with a mean value of 22.6 mg. as determined for 14 men, 20 to 36 years of age. Since the details of this work have not appeared, it cannot be appraised.

The mean values reported for adult women (table 3) do not show quite such large discrepancies as those for men. If the low values of McCullagh *et al.* and Callow are disregarded, the range of mean values for young women is approximately 7 to 12 mg. per day with the lower limit of normal at about 3 mg. and the upper limit at about 22 mg/24 hours. Eight of 14 mean values determined with the aid of various modifications of the Zimmermann reaction lie between 9 and 12.2 mg. per day. In contrast to the values reported by him for men, Dorfman (69) reported for 30 women 20 to 40 years of age, a mean value of 12.6 mg. with a range of 4.0 to 22.0 mg. per day. This mean value is of the same order as most of those included in table 3. It is interesting that Salter *et al.* (215) found in the case of women no significant differences between the mean value determined by the method of Cahen and Salter and

that determined by the Pincus method. It would appear from this result that the 17-ketosteroids excreted by women are subject to less quantitative and qualitative variations than those of men.

The variation in the mean values is somewhat disturbing in the case of men. However, the tabulated figures give a general picture of the range of values which may be expected.

The variation which has been discussed makes it desirable for each laboratory to set up its own standard of normal values. If this procedure is not practical, a consideration of the ranges of values of tables 2 and 3 will show with reasonable certainty whether or not a given value is abnormally high or low. The lowest values for women obtained by McCullagh *et al.* and Callow *et al.* (1.5 and 1.7 mg. per day, respectively) are out of line with all of the other values and are of the order of magnitude usually considered to be associated with Addison's disease. It appears to be unnecessary to accept them as truly normal values. Also, there is no doubt that some normal men may excrete much less than 13.1 mg. which is the lowest value found by Salter *et al.* in 1948 (217).

Variations in Output of 17-Ketosteroids in Normal Adults. The rate of excretion of 17-ketosteroids by an individual is not absolutely constant during 24 hours nor from day to day. Evidence on the effect of change in urine volume is conflicting. Pincus (200) observed that the rate of excretion of 17-ketosteroids was higher during the day than during the night; also, in general the rate of formation of urine was less during sleep. However, forced diuresis did not significantly alter the excretion of 17-ketosteroids by one subject. Similar diurnal variations in other urinary constituents have been observed (158, 206). Thus, the exact relation between volume of urine and excretion of 17-ketosteroids cannot be defined. At least, in the experience of Pincus, diuresis per se was not the cause of the increased excretion of 17-ketosteroids during waking hours. Bachman *et al.* (5) also found evidence of the independence of the volume of urine and ouptut of 17-ketosteroids by women. However, McHenry *et al.* (157) presented data which indicated a statistically significant relation between the per diem volume of urine and the amount of 17-ketosteroids excreted. Also, they found excellent correlation between the specific gravity of the urine and the concentration of 17-ketosteroids. Wooster (278) found that extreme diuresis during two days produced on the first day a significant increase in the excretion of 17-ketosteroids which was followed by a decrease on the following day. In view of the many physiologic variables which may affect the excretion of 17-ketosteroids, ordinary variations in the volume of urine probably can be ignored. Forbes *et al.* (85) were unable to find any relationship between excretion of 17-ketosteroids and height, weight, complexion or excretion of creatinine.

Diurnal variations in the excretion of 17-ketosteroids also were observed by Callow *et al.* (31). Pincus found that the rate of excretion was 30 to 50 per cent greater during waking hours than during sleep with the maximal rate in the morning; also, high night titers appeared to be associated with high day titers. Forbes *et al.* made similar observations although their variations were somewhat different. The rate of excretion during sleep was 22 per cent less than the rate of excretion during the waking hours and the rate during sleep was almost twice as constant. These observa-

tions make it desirable to collect urine over a 24-hour period in order to smooth out the variations and obtain a representative sample. Reifenstein (210) suggested use of the 'basal excretion' which is the amount excreted during sleep multiplied by 24 and divided by the hours of sleep. Wooster (278) found that such a basal excretion did not correlate with the complete 24-hour excretion and concluded that analysis of either basal specimens or 12-hour night specimens to estimate the total 24-hour excretion of 17-ketosteroids is inaccurate and unreliable. On the other hand, Bachman, Leekley and Winter (5) found no significant difference in the 17-ketosteroid content of urine excreted at night and that excreted during the day. It does not seem possible to reconcile these conflicting results, but it appears to us that a diurnal variation in the excretion of 17-ketosteroids is the usual pattern.

Most workers have adopted 24 hours as the standard period for collection of urine specimens. A longer period undoubtedly would give a more accurate picture of the excretion of 17-ketosteroids but usually this is not practical. Rather, at times, it may be necessary to use a shorter period. Talbot and Butler (239) estimated that a single determination on a 24-hour specimen would give a value within 15 per cent of the mean value obtained by repeated determinations. Werner's (264) estimate was ±40 per cent and Chou and Wu's (36) ±20 per cent. Drekter *et al.* (74) found that the average deviation from the mean value for an individual was less than 30 per cent in the majority of cases and never more than 36 per cent. Wooster (278) found, in a study of one medical student, that the variance in consecutive values was substantially the same as the variance among single determinations on a group of 10 medical students. It was concluded that for the purpose of screening patients for a possible abnormal excretion of 17-ketosteroids one or two determinations are adequate. Miller, Mickelson and Keys (176) observed considerably smaller variations than those just mentioned. They found that 2 to 5 samples secured from each of 12 subjects 7 to 81 days apart showed an average deviation of 5.5 per cent from the mean 24-hour excretion of the individual. Each individual seemed to have a characteristic level of excretion.

Although variations in the excretion of 17-ketosteroids by normal individuals may not be very large in terms of percentage, in the same terms much greater fluctuations are seen when the level of excretion is very low, and occasionally when distinctly elevated levels are encountered.

Older Adults. Hamilton and Hamilton (116) studied the effect of aging on the excretion of 17-ketosteroids by physically normal men who were prison inmates. It will be observed that their data in table 2 show a progressive decline with age in the amounts of 17-ketosteroids excreted. By the seventh decade the average amount is only half the average amount excreted by men in the third and fourth decades. There was indication of a high degree of correlation between excretion of 17-ketosteroids and body weight, a finding which is contrary to that of Forbes *et al.* (85). In the third and fourth decades the mean values found by Hamilton and Hamilton (10.8 and 10.1 mg.) were lower than the mean value of a group of 8 men, 18 to 47 years of age, who were not prison inmates. Although it appeared that the amounts of 17-ketosteroids may have been diminished by the prison environment, a good reason could not be found for this effect. However, the physical activities of the men were

slight during the period of study. As the total 17-ketosteroids excreted decreased with age there was no great relative increase in the amount of either the α or β fraction that would suggest compensatory activity of the adrenal cortices in response to any loss of testicular function. A few determinations scattered through the literature (tables 2 and 3) also show a lowered excretion of 17-ketosteroids by men and women past the age of 60.

Contrary to the report of Hamilton and Hamilton, Wooster (278), in a study of 56 male prisoners, 20 to 60 years of age, found no significant changes in the mean levels of excretion with age, but the younger men had a greater variance. In contrast to the observations of Forbes *et al.*, there was a significant correlation between excretions of 17-ketosteroids and total creatinine. An interesting observation was that prisoners convicted of violent crimes excreted more 17-ketosteroids than those convicted of nonviolent crimes. This difference decreased with age. It will be noted (table 2) that the mean values of Wooster for the prisoners 20 to 39 years old were similar to those of Hamilton and Hamilton for prisoners of this age group and that they support the suggestion that prison life results in a lowered excretion of 17-ketosteroids.

Forbes *et al.* (85) concluded that in women the normal level of excretion decreases somewhat with age but not markedly until after the climacteric. This conclusion agrees with the observations of Hamblen and associates (113). Hamburger (115) found in a study of 137 normal males (3 to 102 years of age) and 127 normal females (2 to 92 years of age) that the excretion of 17-ketosteroids reached a maximum in both sexes at 25 years of age and the average at that time amounted to 15.4 mg. per day for males and 10.0 mg. per day for females. In the male the average output gradually fell to senescence while in women there was an abrupt fall in the average output from the ages of 25 to 35 but thereafter the rate of excretion remained at a plateau until the sixth decade when a gradual decline ensued.

Although the evidence is somewhat conflicting, it seems established that the excretion of 17-ketosteroids of men and women tends to decrease with age but that there is not a significant decrease, that is, to values below the range for normal young adults, until the age of 50 to 60 years. In individual cases this value may not decrease below the lower limit of the normal range of values for younger individuals. The two subjects of Venning and Kazmin (259), 77 and 81 years of age, excreted 10.0 and 10.6 mg. per day, respectively (table 2). These values are just at the lower limit of the range of normal values for young men as established by Venning and Kazmin.

The lowered excretion of 17-ketosteroids in the later years of life suggest diminished testicular and adrenal cortical production of steroid hormones although a change in the metabolic disposal of these hormones is also a possibility. The observation, which will be treated more fully later, that removal of the gonads led to an increased excretion of 17-ketosteroids, prompted the suggestion that "the adrenals become the gonads of the aged." The data at hand on the excretion of 17-ketosteroids by intact aged individuals do not support such an idea.

Children (0 to 18 Years of Age). Data on the excretion of 17-ketosteroids of children from early childhood to late puberty have been collected in tables 4 and 5.

Talbot *et al.* (241) found no significant differences in the excretion of 17-ketosteroids of the two sexes up to 18 years of age and therefore made no distinction in the published values. The figures of Raices and Rivara (208) showed a similar correspondence of the mean values up to age 12; after that there appeared to be a significant increase in the mean values for the males over the mean values for the females, although the number of subjects in this age range is small. The figures of Miller and Mason (177) which covered the range of ages from 12 to 17 years showed little if any divergence of the mean values for the two sexes up to age 17.

The values of Raices and Rivara are considerably higher at all ages than those of Talbot *et al.*, whereas the limits of Miller and Mason agree well with those of the

TABLE 4. URINARY 17-KETOSTEROIDS OF PERSONS LESS THAN 20 YEARS OF AGE, MG/DAY

AGE, YR.	BOYS						GIRLS						BOYS AND GIRLS[1] REFS. (239, 241)	
	Ref. (208)			Ref. (177)			Ref. (208)			Ref. (177)				
	No.	Range	Mean	No.	Range	Mean	No.	Range	Mean	No.	Range	Mean	Range[2]	Mean
3							4	0.0–1.8	0.6				0.0–0.3	0.15
4	3	0.0–1.3	1.2				8	0.0–2.1	1.2				0.0–0.5	0.3
5	7	0.0–2.4	1.8				6	0.0–2.4	1.4				0.0–0.7	0.4
6	4	1.0–3.0	2.0				9	0.5–3.9	3.0				0.0–1.0	0.5
7	4	1.6–3.7	2.6				6	0.5–4.6	3.2				0.0–1.4	0.65
8	3	2.6–4.3	3.9				7	0.7–6.8	4.0				0.0–2.0	0.95
9	4	3.1–5.9	5.0				17	1.2–6.8	5.5				0.2–2.8	1.4
10	5	6.7–8.1	7.6				15	2.0–9.6	7.2				0.5–3.6	1.9
11	7	4.8–12.8	9.3				10	6.1–10.4	8.4				1.2–4.3	2.6
12	7	7.6–12.8	11.0	9	1.0–2.3	1.8	5	5.2–12.5	8.7	7	1.6–5.3	2.9	1.7–5.5	3.4
13	4	9.4–14.9	13.1	6	0.9–8.0	3.4	4	8.8–12.6	11.0	9	1.0–5.6	3.0	2.4–8.2	4.3
14	2	14.0–14.2	14.1	17	3.3–15.6	6.3	4	6.8–16.7	12.8	9	3.6–10.0	6.0	3.1–8.0	5.3
15	2	16.0–16.2	16.1	9	4.1–11.3	6.9	3	9.8–14.8	13.4	7	3.5–9.9	5.5	3.6–9.0	6.3
16				8	3.4–10.5	7.6				10	3.8–13.1	7.5	4.2–10.2	7.2
17				7	3.7–12.7	8.5				16	3.3–9.8	6.6	4.9–11.5	8.1
18				1	6.0								5.5–12.5	8.6

[1] 69 boys and 31 girls.

[2] These values are taken from a chart (239) and are subject to an error of ±0.1.

latter authors. The fragmentary values of Venning and Kazmin (259) (table 5) are even higher than those of Raices and Rivara in the age range of 3 to 7 years and those of Hain (106) (table 5) tend to agree better with the values of Raices and Rivara than with the values of Talbot *et al.* The reason for these discrepancies is not apparent; differences in techniques employed by the different investigators may afford a partial explanation. In the case of very young children it might be suggested that actually very little 17-ketosteroids are excreted but that nonsteroid chromogens have given falsely high values. Talbot *et al.* pointed out that their values were lower than those of Oesting and Webster (190) and of Nathanson *et al.* (184) and also lower than their own earlier figures. They attributed their lower values listed in table 4 to the use of the correction equation which was introduced to reduce the errors resulting from interfering chromogens. Miller and Mason also corrected

their readings by means of this equation. However, the discrepancies are still large for older children and in this group the effects of the nonsteroid chromogens probably would not account for the differences. Perhaps racial or environmental differences may account for the differences in the amounts of urinary 17-ketosteroids as determined by Talbot *et al.* who worked in Boston, and by Raices and Rivara, who worked in Argentina.

Nathanson *et al.* (184) published values for children 3 to 15 years of age which are of the same order of magnitude as those of Raices and Rivara although somewhat higher in the age group of 3 to 7 years. They used Oesting's method and found, as did Raices and Rivara, that excretion of 17-ketosteroids by boys increased more rapidly after 11 years of age than the excretion by girls.

Day (49) (table 5) found that newborn babies excreted small but definite amounts of 17-ketosteroids as determined by the method of Cahen and Salter.

It is our opinion that the available data indicate that excretion of 17-ketosteroids by children up to age 6 or 8 years is likely to be very small and frequently less than one mg. per day. The amounts excreted, by boys and girls alike, then increase slowly to the age of puberty. After that age the increase, which may be greater for boys than for girls, is more rapid and average adult levels are approached, but not reached, by 17 years of age. Very few determinations have been made on persons in the period between the ages of 17 and 20. Considering the results of Talbot *et al.*, of Hamburger, and of Miller and Mason, it seems that there must be a considerable increase, on the average, in the amount of 17-ketosteroids excreted during this period in order to account for the greater mean values found in the 20- to 30-year age group. As previously noted, in the large series reported by Hamburger, the maximal average excretion for men and women is reached at 25 years of age.

According to Talbot *et al.* (241), in the case of children less than 11 years of age, the low normal rate of excretion permits the easy detection of an abnormally high output of 17-ketosteroids whereas the detection of hypo-excretion is impossible. However, by 12 years of age a child should excrete at least one mg. of 17-ketosteroids per day.

Since the adrenal cortex and testes are the only known sources of urinary 17-ketosteroids, the similar amounts excreted by boys and girls suggest that the adrenal cortex is the chief source of these substances during childhood. To quote Talbot *et al.* (241), "If this is so, the fact that the output of 17-ketosteroids rises as a person changes from a child to an adult implies a relation between the secretory activity of the adrenal cortex and growth and development. Moreover, the continued rise in values for the excretion of 17-ketosteroids from 14 to 18 years suggests that the menarche in girls and its equivalent in boys do not necessarily culminate the endocrine development which started several years earlier."

β-Fraction. The proportion of the β-fraction present in the 17-ketosteroids excreted by normal individuals appears to be subject to as much variation as the total 17-ketosteroids. Part of the variations reported may be attributable to differences in the methods used. The part played by methods, and particularly the method of hydrolysis, has been discussed. The β-fraction normally does not exceed 15 per cent of the total 17-ketosteroids as it is determined by methods in current use.

EFFECTS OF CHANGES IN THE ENDOCRINE GLANDS

Pituitary. Much evidence has accumulated that the function of the adrenal cortex is directly controlled by a trophic hormone produced by the anterior pituitary gland. It has already been pointed out that administration of this adrenocorticotrophic hormone to human subjects causes an increased excretion of 17-ketosteroids as well as an increased excretion of corticosteroids. Conversely it is well known that the adrenal cortices of hypophysectomized animals undergo atrophy. Since gonadal function also is under pituitary control, it would be expected that pituitary insufficiency from any cause would be associated with a marked decrease in the excretion of 17-ketosteroids by both sexes. This expectation is supported by the data of table 6.

Tumors of the anterior pituitary body produce variable effects on the excretion of 17-ketosteroids. The level of excretion depends on the nature of the lesion and the stage of the disease. Chromophobe adenomas appear to produce endocrine disturbances only in so far as they destroy the normal function of the gland. The degree of insufficiency is variable; in some cases it is barely detectable, in others it is severe. Similarly the 17-ketosteroids excreted vary; in some cases appreciable quantities of these steroids are excreted in the urine, in others they are virtually absent (78). Eosinophilic adenomas associated with acromegaly usually produce their endocrine disturbances slowly. Early in the disease, in addition to the acromegalic features, diabetes, hypermetabolism and goiter, some hypertrichosis and hypertrophy of the skin may appear. In the late stages evidences of pituitary insufficiency may supervene and many of the early symptoms diminish in severity. It is not surprising that a disorder which runs such a complex course and which may show such widespread lesions is associated with variable excretions of 17-ketosteroids. The values given in table 7 range from subnormal to values several times the upper limit of normal. Unfortunately, adequate clinical and pathologic data are not available in these cases. One can only assume variable activity of the anterior pituitary and of the peripheral endocrine organs. The results of Mason and Sprague (171), in 35 cases of acromegaly, are in essential agreement with the data of table 7. When the patients were females (19 cases), the range was 2.7 to 21.0 mg. per day; in 15 cases the values were within their normal range (4 to 17 mg.). When the patients were males (16 cases), the range was 0.7 to 28.4 mg. per day; in 7 cases the values were within normal limits (6 to 21 mg.) and in 6 cases they were below the lower limit of the normal range.

Thyroid Gland. The thyroid hormone has such profound effects on all bodily functions that it is not entirely unexpected to find a diminished excretion of 17-ketosteroids in cases of myxedema. The amounts of 17-ketosteroids excreted may vary from zero to the lower limits of normal (table 8) and are frequently less than 2 mg. per day. An interesting but unexplained difference has been observed between cases of spontaneous myxedema and myxedema of short duration resulting from thyroidectomy or thyroiditis. Engstrom and Mason (80) reported that in 2 of the former cases, in which extremely small amounts were excreted, there was no rise in the excretion of 17-ketosteroids, and in 3 at most, an equivocal slight rise when desiccated thyroid was administered in sufficient amounts to relieve the myxedema. In none of these cases did the rate of excretion return to normal levels. In cases of

myxedema of short duration, however, relief of the myxedema was accompanied by return of the excretion of 17-ketosteroids to normal levels.

Friedgood (92) also reported very low excretion of 17-ketosteroids in cases of myxedema (table 8), but adequate thyroid replacement therapy did not restore the 17-ketosteroid excretion to normal either in spontaneous or post-thyroidectomy myxedema. It may be significant that 4 of Friedgood's 5 patients had been receiving thyroid therapy for 13 months to 10 years at the time the values for 17-ketosteroids were determined. Engstrom and Mason and Friedgood reviewed some of the possible factors involved in the effect of myxedema on the excretion of 17-ketosteroids. There is no adequate explanation of the observations just discussed.

Talbot *et al.* (241) observed that the excretion of 17-ketosteroids by hypothyroid children who were receiving sufficient thyroid to eliminate symptoms of hypothyroidism was slightly below the normal range for children of corresponding ages. The 'developmental' ages of these children also were retarded below their chronologic ages. Benda and Bixby (11) reported that 2 female cretins 23 years of age excreted 2.7 and 3.3 mg. per day and one male cretin 30 years of age 5.4 mg. per day. Necropsy in 2 cases revealed gonadal and adrenal infantilism, conditions which are compatible with the low values for the 17-ketosteroids.

Hyperthyroidism is associated with a somewhat decreased excretion of 17-ketosteroids (80). This decrease is evident in the mean values but the individual values lie within the normal range. Possibly the slight alterations from normal can be accounted for by nonspecific secondary effects of the illness.

Diabetes Mellitus. In controlled diabetes mellitus Miller and Mason (177) found that the average excretion of 17-ketosteroids was decreased; in some cases the values were below the lower limit of normal. In the majority of cases, however, the values were within the normal range. In one instance the value was somewhat above their upper limit of normal. If the diabetes should be out of control, the effect of stress on the excretion of 17-ketosteroids must be considered. Diabetes mellitus is seldom an uncomplicated metabolic disorder. Most chemical measurements in this disease have a wide range of variability and mean values have a tendency to diverge in one direction or other from the normal, for example, those for serum albumin and serum cholesterol.

Testes. It is generally agreed that the interstitial cells (Leydig cells) of the testis produce the androgens which contribute to the urinary 17-ketosteroids. Other factors remaining equal, failure of these cells to function may be expected to reduce the amount of 17-ketosteroids excreted. However, complete failure of this testicular function in an individual may not reduce this amount to levels below the normal range. There are many possible complications, however, and a knowledge of the genesis of the failure is of utmost importance before interpretation of the values for 17-ketosteroids can be made. The origin of the failure is frequently in doubt except after castration or when a distinctly local testicular disease is present. In individual cases it is often difficult to distinguish 'primary' failure (a genetic or other defect in the germ plasm) from a 'secondary' failure (one resulting from failure of anterior pituitary gonadotrophic function or from nutritional deficiencies). A deficiency that originates prior to puberty may not produce the same effect on the excretion of 17-

ketosteroids as a deficiency that occurs after puberty. Gonadal failure consequent on pituitary or even other general diseases is frequently associated with altered function of other endocrine glands such as the adrenal cortex or thyroid, and altered function of these glands alone may alter the excretion of 17-ketosteroids. Hence, a normal value for the urinary 17-ketosteroids would not rule out failure of androgen elaboration by the testes nor would a low value prove a primary gonadal failure.

Determination of the amount of 17-ketosteroids excreted in cases in which there is clinical evidence of failure of the testicular androgenic function, regardless of the

TABLE 5. MISCELLANEOUS VALUES FOR URINARY 17-KETOSTEROIDS OF NORMAL CHILDREN

AGE	17-KETOSTEROIDS, MG. PER DAY					REFERENCE
	Boys			Girls		
	No.	Range	Mean	No.	Range	
Hours						
24–96	7	0.13–0.67	0.35			(49)[1]
Days						
1–4		<1.0				(259)[2]
Years						
2	1	0.6				(106)[3]
3	3	2.0–2.5				(259)[2]
5				1	1.4–2.5 (2 detns.)	(106)[3]
5.5	1	5.5				(259)[2]
6.5					2.2	(106)[3]
7	1	4.5		1	1.8	(259)[2]
8	1	4.1		2	2.6–7.1	(106)[3]
8.5	1	2.4		2	1.3–2.0	(106)[3]
9		3.6		2	2.2–2.7	(106)[3]
9.5		3.0				(106)[3]
11.5	1	2.0–5.3 (6 detns.)	3.6			(106)[3]
13	1	6.0				(259)[2]
14		6.0				(106)[3]
16	1	10.0				(259)[2]

[1] Method of Cahen and Salter (24) [2] Method of Holtorff and Koch (132) [3] Method of Patterson *et al.* (193).

genesis of the condition, gives values which range from below normal to high normal. Werner (267) reported values ranging from 2.6 to 14.6 mg. for 16 'eunuchoid' patients; two-thirds of the patients excreted amounts within his normal range (7 to 17 mg/24 hours). These results may be considered to be representative (28, 29, 119). In such cases the adrenal cortex presumably is the source of the 17-ketosteroids. In the cases of Klinefelter *et al.* (144) syndrome characterized by gynecomastia and aspermatogenesis the Leydig cells are intact and the excretion of 17-ketosteroids is normal or, in some instances, somewhat low (118, 119).

Evidence of reciprocal relations of the adrenal cortex and the testes in laboratory animals has been provided by a number of investigators (18, 42, 46, 51, 99,

110), but the conclusion which might be drawn from these studies, that removal of the testes may be compensated for partly by increased production of androgens and 17-ketosteroids by the adrenal cortex, is not necessarily applicable to the human species. The evidence with respect to human beings is somewhat conflicting although there is some evidence that an increased production of 17-ketosteroids by the adrenal cortex does occur after castration.

Callow, Callow and Emmens (29) found that the excretion of 17-ketosteroids by 11 eunuchs was within the range of normal but that the average value was less than the average value for normal persons. These results have been confirmed by others. However, McCullagh *et al.* (156) reported that 5 eunuchs excreted amounts which were about one-half the average of normal persons in their series.

More impressive results have been obtained by study of the excretion of 17-ketosteroids before and after surgical castration. In 10 cases (ages 47 to 80 years) of prostatic cancer, Scott and Vermeulen (226) observed that the 17-ketosteroid excretion fell in all cases after orchiectomy but the fall was not sustained. In all but one case excretion of 17-ketosteroids gradually increased to levels higher than before castration. In only one case was there an increase in the β-fraction. Frame and Jewett (89) obtained similar results. The obvious interpretation of these results, namely, that the production of 17-ketosteroids by the adrenal cortex was increased as a consequence of castration, does not necessarily apply to men who are otherwise normal save for loss of the testes. The subjects of Scott and Vermeulen and of Frame and Jewett were suffering from malignant disease and the presence and severity of the disease before and after castration may have had an influence on the production of 17-ketosteroids by the adrenal cortex or on their metabolism, or on both processes.

Callow and Callow (28) isolated the ketonic steroids from the urine of a eunuch. Compared with the amounts isolated from normal urine, they isolated somewhat smaller amounts of androsterone and etiocholanolone but much larger (10 times) amounts of dehydroisoandrosterone. The total amount of ketonic steroids isolated was slightly greater than the amount isolated from normal urine. It should be noted that the total amount isolated in either case was only a small fraction of the 17-ketosteroids as determined colorimetrically. The increased amount of dehydroisoandrosterone suggested the possibility of increased adrenal cortical activity with respect to the 17-ketosteroids. However, the 17-ketosteroids of one eunuch were compared with the pooled 17-ketosteroids of a number of normal men. In view of individual variations such a comparison is hardly valid.

In a study of the excretion of hypophyseal gonadotrophin, estrogens and 17-ketosteroids, Werner (266) was unable to find any correlation in the amounts of these substances excreted by 4 normal men. In view of the relatively minor contribution of the testes to the total 17-ketosteroids (see table 10) it is not likely that moderate changes in the amounts elaborated by the testis could be detected, even on the assumption that the amount of urinary gonadotrophin reflects in some measure the degree of testicular stimulation by this hormone.

Interstitial cell tumors of the testis are very rare. The one case studied by Venning, Hoffman and Browne (258) has been mentioned. Androsterone sulfate was

isolated from the unhydrolyzed urine and after hydrolysis of conjugates, androsterone, etiocholanolone and isoandrosterone were isolated (131). Androsterone comprised the major portion of the 17-ketosteroids isolated. Acid hydrolysis of the androsterone sulfate also yielded some $\Delta^{(2 \text{ or } 3)}$-androsten-17-one.

Ovaries. Although there is some equivocal evidence that normal human ovaries can produce small amounts of androgen, their contribution to the amount of 17-ketosteroids excreted under normal circumstances is certainly very small and is usually ignored. During normal menstrual cycles there is no evidence of cyclic variations in the excretion of 17-ketosteroids. The small fluctuations which occur bear no relation to the cycle (54, 265). Nevertheless, it is not possible to conclude that ovarian activity does not influence adrenal function under any circumstances (146).

The potentialities of the ovary for producing androgens become evident in the presence of some ovarian tumors. Arrhenoblastomas are strictly virilizing tumors. Studies of the excretion of 17-ketosteroids have been made in only a few cases of arrhenoblastoma. Four of the tumors mentioned in table 9 were definitely classed as arrhenoblastomas. In only one of these 4 cases, that of Jones and Everett (137) was the excretion of 17-ketosteroids greater than normal; in the other 3 cases the values were in the normal range or below. Possibly these tumors produce androgens which are not metabolized to 17-ketosteroids. The nature of the urinary steroids which are excreted by patients with arrhenoblastomas has not been studied.

Adrenal-like ovarian tumors presumably arise from aberrant adrenal cortical cells in the ovary and would be expected to produce consequences similar to those produced by adrenal cortical tumors. Only 4 authentic cases of this type are listed in table 9. In 2 of these excessive amounts of 17-ketosteroids were excreted, in 2 cases normal amounts. This difference is quite in keeping with the differences in the excretion of 17-ketosteroids in cases of adrenal cortical tumor. No specific pathologic data are available in 3 of the reported cases of virilizing ovarian tumors. All 3 patients excreted excessive amounts of 17-ketosteroids.

Hamblen and associates (111, 112) have studied the excretion of 17-ketosteroids associated with various menstrual disturbances. Since their determinations were made with Oesting's method, the results can be compared only qualitatively with later ones. In 8 cases of 'delayed menarche,' associated with physical signs of estrogen deficiency, values for urinary 17-ketosteroids were normal. In 23 cases of 'intercurrent amenorrhea' the excretion of 17-ketosteroids increased when the amenorrhea had lasted 6 months or more. It was suggested that "late or intercurrent ovarian failure precipitates androgenic hyperfunction of the adrenal cortex." Menometrorrhagia related to failure of the corpus luteum was accompanied by normal values for the 17-ketosteroids. It was concluded that "progesterone plays no active part in the reciprocities of function of the gonadopituitary and adrenopituitary axes."

Hamblen *et al.* (113) also reported that recently ovariectomized women excreted normal amounts of 17-ketosteroids but women who had been ovariectomized 7 to 8 months previously excreted increased amounts. In the case of women who had undergone a normal menopause the amounts excreted were increased during a 5-year period but had decreased to a normal level after 14 to 26 years. However,

these data are at variance with other studies. Callow, Callow and Emmens (29) found normal values in the case of ovariectomized women but the average was below the normal average value. Raices (207) found that in the menopause the values were within the limits of normal and Hamburger (115), in a large series of cases, noted no remarkable fluctuations in excretion of 17-ketosteroids at the menopause and only observed a gradual decline in steroid excretion after the sixth decade of life.

Hirschmann (125, 126) isolated the ketonic steroids from the urine of ovariectomized women and was able to isolate the same compounds that Callow and Callow (27) obtained from the urine of normal women, and in comparable amounts. Clearly, more precise data are needed in the case of women on the relationships which might exist between the excretion of hormones, aging processes and normal and abnormal ovarian function.

Pregnancy. Venning (256), using both the Holtorff-Koch modification of the Zimmermann reaction and the Pincus method, found that, whereas the chromogenic material active in the Zimmermann reaction increased during pregnancy, the material active in the Pincus reaction remained fairly constant. Since the latter method determines only 17-ketosteroids, the results indicate that the total excretion of 17-ketosteroids is not affected appreciably by pregnancy. Since the Zimmermann reaction also responds in some degree to 20-ketones, it appears that the increase of pregnanolones and related 20-ketones is sufficient to account for the apparent increase in 17-ketosteroids when measured by the Holtorff-Koch method. This interpretation is supported in part by a recent report of Dobriner, Lieberman, Rhoads and Taylor (64) who isolated increasingly large amounts of pregnan-3-ol-20-one as gestation progressed. They also found that the amount of androsterone excreted decreased and disappeared by the eighth month and that the excretion of etiocholanolone was very much reduced. 11-Ketoetiocholanolone was excreted in apparently normal amounts but 11-hydroxyandrosterone was absent from the urine. One gains the impression from this isolation work that the total amount of 17-ketosteroids excreted must decrease as gestation proceeds, a conclusion that is at variance with Venning's. However, while it is uncertain whether or not the total neutral 17-ketosteroid fraction remains quantitatively unchanged, it is apparent from the work of Dobriner *et al.* that the qualitative composition of this fraction is strikingly altered during pregnancy.

Adrenal Cortex. Evidence that, in both sexes, the major portion of the urinary 17-ketosteroids is derived from the adrenal cortex has been discussed. Obviously, pathologic changes in the gland which influence its function will be reflected in the amount, and possibly the kind, of 17-ketosteroids excreted in the urine. Because of the wide range in the amounts excreted by normal persons, in the individual case it may not be possible to detect a change in function by a change in the urinary 17-ketosteroids. In many instances, however, an abnormally high or low excretion may give a clue to the nature of the pathologic lesion which is causing the abnormality.

a) Addison's Disease. Consistently low values for the urinary 17-ketosteroids have been observed in cases of Addison's disease (table 10). The values are

frequently less than one mg/24 hours and are sometimes zero. Because of the contribution of the testes in men, the lowest values are observed in the case of women. There is some discrepancy in the results of different investigators. For example, Fraser *et al.* (90) consistently obtained lower values than did Callow *et al.* (29) or Venning and Browne (257). These variations probably result from differences in the techniques used for the chemical assay. Fraser *et al.* used the technique of Callow *et al.* but compensated for the presence of interfering chromogens by the use of a correction equation. Callow *et al.* recognized that many of their values were too high since atypical colors developed in certain crude extracts. Although Venning and Browne applied a correction in their use of the Holtorff-Koch method, higher values than those of Fraser *et al.* might be expected. Differences in technique which are not readily apparent when dealing with values that are within or above normal adult levels may become evident when dealing with very low levels of steroid excretion.

The low excretion of 17-ketosteroids in Addison's disease is a consequence of destruction or atrophy of the adrenal cortex. The excretion of 17-ketosteroids may be slightly variable but is almost always low by the time the disease is recognizable.

TABLE 6. ANTERIOR PITUITARY INSUFFICIENCY

REFERENCE	CASES	AGE, YR.	17-KETOSTEROIDS, MG/DAY	REMARKS
(90)	15		0.5–2.8	In 13 cases average values <0.5 mg.
(91)	10		0.0–1.5	In 9 cases, zero values
(92)	5		0.5–2.5	
(171)	32		0.0–3.6	In 17 cases, values <1.0 mg.
(251)	3	39–41	0.0–1.5	All women
(257)	5	32–48	1.4–4.5	

In our experience values of one mg. or less for women and 3 mg. or less for men furnish collateral evidence for Addison's disease. Such evidence alone cannot be considered as diagnostic since there are many other states associated with similarly low levels of excretion. On the other hand, a normal rate of excretion would oppose a diagnosis of adrenal cortical insufficiency, especially in women.

b) ADRENAL CORTICAL TUMORS AND HYPERPLASIA. Tumors and hyperplasia of the adrenal cortex often produce similar signs and symptoms which, in women and children, may vary from extreme virilism (so-called adrenogenital syndrome) to full-blown Cushing's syndrome (facial and abdominal obesity, thin arms and legs, purple striae and ecchymoses, plethora, hypertension, osteoporosis, diminished carbohydrate tolerance, muscular weakness and occasionally polycythemia and hypochloremic alkalosis). These syndromes are attributed to the effects of an overproduction of adrenal steroids by the tumor or hyperlastic cortex. In many cases the picture is a mixed one, with features of virilism and of Cushing's syndrome. 'Over-masculinization' has not been described in adult males suffering from adrenal neoplasm, but feminization has been observed in a few cases, with testicular atrophy, impotence, some loss of body hair and breast enlargement. Since the same clinical picture may accompany either neoplasia or hyperplasia of the cortex, identification of the pathologic lesion often is a difficult problem. Examination of the urinary 17-ketosteroids

may afford some help in this problem, but unfortunately too often the results are equivocal.

Cortical tumors. Tumors of the adrenal cortex almost always are associated with increased amounts of urinary 17-ketosteroids (table 11). However, exceptions are noted (*cases 14* and *32*). One of us (Mason) has recently encountered 2 cases of severe Cushing's syndrome associated with adrenal cortical tumors in which the urinary 17-ketosteroids were 3.5 to 7.3 mg. and 3.2 to 5.7 mg. per day, respectively. The patients were women, 25 and 43 years of age. These values vary from the low normal range to below the lower limit of normal. In the first case evidence of cortical hyperfunction was found in increased excretion of corticosteroids. In the other case excretion of corticosteroids was within normal limits. In this case then there was no evidence in the quantity of urinary steroids of the overproduction of adrenal steroids which must have occurred in order to produce the clinical picture. Rarely, a cortical tumor may be nonfunctioning, as far as can be determined, and therefore does not influence the 17-ketosteroid excretion (*case 30*, table 11).

In cases of tumors with signs and symptoms of adrenal cortical hyperfunction, the urinary 17-ketosteroids have varied from a few milligrams, in children, to more than 1000 mg. per day in adults. The data now available indicate that in the case of women a value over 150 mg. per day is diagnostic of the presence of an adrenal cortical tumor. At any rate, no other condition has been reported which is associated with values above this level.

Determinations of the 17-ketosteroids have been made in only a few cases of functioning tumor in which the patients were adult males. It may be noted that all of the 4 values in table 11 are less than 100 mg. but greater than 40 mg. per day. An interstitial cell tumor of the testis and adrenal cortical hyperplasia also must be considered when there is an increased value of the 17-ketosteroids in males.

None of the adult males in table 11 showed definite evidences of feminization although one patient (*case 21*) was reported to be impotent and to possess small soft testes. Two of the adult male patients (*cases 16* and *21*) presented the picture of Cushing's syndrome and 2 (*cases 38* and *40*) showed no evidence of endocrine or metabolic abnormalities in spite of the fact that in *cases 38* and *40* the urinary 17-ketosteroids were increased to levels well above the normal.

Among children extremely variable results have been found but it is seen that even the lowest values are several times greater than the normal values for children of corresponding ages. The two youngest children, aged $1\frac{1}{4}$ years, exhibited the lowest values, but 2 children, aged $3\frac{1}{2}$ years, excreted at least 170 mg. per day.

In only 16 of the 50 cases was the 3(β)-alcoholic fraction determined. In all but 4 instances this fraction was distinctly elevated, the proportion being 30 to 87 per cent of the total 17-ketosteroids. The exceptions are important since they indicate that an adrenal cortical neoplasm may be present in spite of a normal (*cases 1* and *15*) or almost normal (*cases 16* and *20*) value of the β-fraction.

It must be concluded from all these available data that in cases of cortical tumor there is no evident relation between the total amount of the urinary 17-ketosteroids, the β-fraction, or any particular urinary steroid and the signs and symptoms displayed by the patient.

Cortical hyperplasisa[3]. In the case of females in which masculinization alone (but not Cushing's syndrome) is associated with adrenal cortical hyperplasia, there appears to be a difference between those in which evidence of hyperplasia develops before puberty and those in which it develops after puberty in so far as the 17-ketosteroids are concerned. However, as will be noted later, the precise status of the adrenal cortex is much more uncertain in cases of 'postpubertal virilism' than in cases of 'prepubertal virilism.' Nevertheless, it seems that greater amounts of 17-ketosteroids are excreted by patients with the latter condition. In table 12 are summarized the values recorded in 29 cases (*cases 51* to *79*) of prepubertal virilism associated with proved cortical hyperplasia, adrenal tumor having been ruled out, or with the anatomic abnormalities of the female pseudohermaphrodite wherein cortical hyperplasia is highly probable. The data are arranged according to the chronological age of the patient at the time of assay. Some of these virilized girls were categorized as feminine pseudohermaphrodites since they were born with the genital abnormalities characteristic of this condition. The older individuals had symptoms that dated back to childhood and in addition to pronounced hirsutism, the majority had deepening of the voice, enlargement of the clitoris, and hypoplasia of the uterus, but without other gross genital abnormalities. None of these patients had the stigmata of Cushing's syndrome.

It is apparent that some of these patients excrete more 17-ketosteroids than do some of the patients with adrenal cortical tumors. Values between 50 and 100 mg. are common, particularly for the older individuals. It is, therefore, impossible, in many instances, to distinguish between the presence of hyperplasia and of tumor on the basis of the quantity of 17-ketosteroids excreted. The amount of steroids excreted appears to increase with the age of these patients. The highest values are seen only in the older and the lowest only in the younger age groups. Although chance distribution and selection of cases may account in part for this relation, *cases 64* (second test) and *75* (second test) bear out this impression. These 2 cases were studied by the same investigators one and two years after the initial determinations and the later values were increased 103 and 64 per cent, respectively, over the initial values.

Prepubertal cortical hyperplasia in the male may be associated with precocious somatic and homologous sexual development. In these cases the excretion of 17-ketosteroids is much greater than that for a normal boy of the same age. It may be added that precocious development is not necessarily an indication of adrenal cortical dysfunction.

Postpubertal adrenal cortical hyperplasia may be associated with the development of virilism in women. Data are given in table 13 for the excretion of 17-keto-

[3] For the purposes of this discussion the term 'adrenal cortical hyperplasia' will be used. Generally this term is used in a very loose clinicopathologic sense to denote bilaterally enlarged adrenal cortices associated with such clinical states as female pseudohermaphroditism, some other states of virilism and Cushing's syndrome. Similar clinical findings may occur with adrenal neoplasms. Admittedly, these states are quite different from one another physiologically and clinically. But until the precise nature and genesis of these cortical dysfunctions are elucidated the term 'hyperplasia' can have meaning only to differentiate a non-neoplastic from a neoplastic cortical lesion. From this point of view the term is used here.

steroids in some 131 cases of postpubertal women who had mild or moderate virilism. In these cases adrenal cortical tumor presumably has been excluded but in most of them the precise anatomic and functional status of the adrenal cortex remains uncertain. None had the stigmata of Cushing's syndrome; mild to severe hirsutism was the common feature in all cases; less commonly irregular menses or obesity and rarely amenorrhea were observed. These cases were classified by different investigators under somewhat different terms, for example, 'hirsutism,' 'adrenal virilism,' 'simple hirsutism,' 'postpubertal virilism,' 'familial hirsutism,' We realize that to categorize all of these patients as suffering from adrenal cortical hyperplasia,

TABLE 7. ACROMEGALY

REFERENCE	CASE NO.	SEX	AGE, YR.	17-KETOSTEROIDS, MG/DAY	REMARKS
(90)	59	F	43	2.9 (av.)	Symptoms 15 yr.
	62	F	41	3.6 (av.)	
	58	F	28	4.5 (av.)	Symptoms 1 yr.
(251)	18	F	60	6.2	
(90)	63	M	31	8.3	Symptoms 10 yr.
	61	F	35	10.2	Symptoms 10 yr.
	60	F	36	10.6	Symptoms 3+ yr.
(257)	23	F	32	11.8	Symptoms 3 yr.
	22	F	54	12.7	Symptoms 15 yr.
(261)	138	F	26	15.1	'Early acromegaly'
	132	F	30	20.4	'Acromegalic'
(257)	24	M	39	24.0; 26.3	Symptoms 14+ yr.
	25	M	28	28.6; 37.6	Symptoms 2 yr.
(261)	110	M	34	38.6	
(227)		F	?	70.0	
		M		110.0	

whatever that term may mean, may be unwarranted. In fact, the functional status of the ovary or of other tissues cannot be properly evaluated in these cases. In any event, it is noted in table 13 that many of the excretion rates for the 17-ketosteroids are within the normal range, but some, about half, are elevated sufficiently to afford a strong suggestion of adrenal cortical hyperfunction. If there is cortical hyperfunction in the former cases, it is not sufficient to increase the urinary 17-ketosteroids appreciably. If no cortical hyperfunction is present, the virilism must originate in some other manner.

In cases of Cushing's syndrome associated with cortical hyperplasia, the excretion of 17-ketosteroids is within the normal range of values, somewhat more often than not. A few values as high as 30 mg. or more have been reported but they are

the exceptions and higher values are exceedingly rare. In one case the patient was a boy, 15 years of age, who excreted 63 to 100 mg. per day; the β-fraction was 10 to 18 per cent of the total. At operation bilateral enlargement and hyperplasia of the adrenal cortices were found (231a).

c) Differentiation Between Cortical Tumors and Hyperplasia. The urinary steroids, nonketonic as well as ketonic, have been studied intensively in cases of tumor and of hyperplasia for some clue that would permit invariable differentiation between these two conditions. On the whole, from this point of view, these studies have been disappointing. Also, it may be added that, in spite of the diverse signs and symptoms which may be associated with either of these conditions, isolation studies have not revealed any single 17-ketosteroid or pattern of 17-ketosteroids which may be correlated with the clinical picture.

Although it already has been noted that the total excretion of 17-ketosteroids does not necessarily differ in the two types of hyperfunctioning lesions, they may be partially distinguished by the fact that particular steroids frequently, but not invariably, appear in greater concentrations in the urine in one or the other. Callow (33) and Crooke and Callow (43) found that dehydroisoandrosterone was present in relatively large amounts in the urine in cases of tumor, whereas it could not be readily isolated in other cases with similar signs but in which a cortical tumor was not present. This observation has been amply confirmed, and further isolation studies (32, 129, 167, 261, 275) have indicated that an important difference between the composition of the 17-ketosteroid fraction in the urine of patients with hyperplasia and those with cortical tumor is the preponderance of dehydroisoandrosterone in the urine of the latter. The significance of dehydroisoandrosterone in relation to the pathologic or normal adrenal cortex still remains obscure. It has been isolated from normal human urine (27, 77) and, therefore, appears to be a normal product of adrenal cortical function (33, 242). It may be a primary product of the adrenal cortex produced in abnormal quantities by the tumor, it may be a by-product of some reaction involved in the production of adrenal cortical hormones, or it may be a metabolic product of some unidentified precursor. In any event, it will be recalled that Mason and Kepler (168, 169) have shown that dehydroisoandrosterone (the main 17-ketosteroid of the β-fraction) is capable of being metabolized to androsterone and etiocholanolone (the main 17-ketosteroids of the α-fraction). There is the suggestion in these studies that dehydroisoandrosterone may be the precursor of the androsterone and etiocholanolone which normally appears in the urine as the result of adrenal cortical function.

The β-fraction of the 17-ketosteroids which are excreted normally may vary from 0 to 15 per cent of the total (9, 87, 92, 141, 202, 242) when urine is hydrolyzed in the usual manner with hydrochloric acid. It may be noted that Bitman and Cohen (14) obtained values for the β fraction within these limits when they used hydrolytic conditions most favorable for preservation of this fraction. The proportion of dehydroisoandrosterone, which is the chief constituent of the β-fraction, also must lie within these limits, and, therefore, determination of the β-fraction provides a convenient means of ascertaining a preponderance of dehydroisoandrosterone. Of the 50 cases listed in table 11. the proportion of the β-fraction was deter-

mined in only 16. However, it might be pointed out that, with 3 exceptions (*cases 12, 16,* and *20*), when the total amount of 17-ketosteroids excreted was over 50 mg. in 24 hours the β-fraction was 48 per cent or more of the total. On the other hand, the limited data of table 12 suggest that the β-fraction is not increased greatly in cases of adrenal cortical hyperplasia even though the total amount of 17-ketosteroids may be more than 100 mg. (*case 77*). Thus, data now available suggest that a daily excretion of more than 50 mg. of 17-ketosteroids, 50 per cent or more of which is β-fraction, indicates the presence of an adrenal cortical tumor. Fine distinctions and sharp lines of demarcation cannot be made. Although it has serious limitations, estimation of the β-fraction is the most valuable tool available for differentiating adrenal cortical tumor from hyperplasia when the value for the total 17-ketosteroids lies within the range common to both conditions (141).

In order to make the story complete a substance that may be more characteristic of cortical hyperplasia than tumor may be mentioned even though it is not a 17-ketosteroid. Butler and Marrian (23) isolated pregnane-3(α),17,20-triol from the urine in 2 cases of hyperplasia and Mason and Kepler (167) isolated it in the 4 cases which were studied (another case has been added subsequently). This substance was found in only one case in which the diagnosis of adrenal cortical tumor was made, but unfortunately the diagnosis in this case could not be confirmed since exploration and postmortem examination were refused. This pregnanetriol deserves further investigation.

In their isolation studies Butler and Marrian (23) as well as Wolfe *et al.* (275), Mason and Kepler (167), Hirschmann *et al.* (127–129) and Lieberman *et al.* (153) did not find any 17-ketosteroid (or other ketosteroid) which could be considered a completely characteristic product of a cortical tumor or of a hyperplastic adrenal cortex.

Male Pseudohermaphroditism. As indicated previously, the direct cause of female pseudohermaphroditism is generally believed to be excessive production of androgen by hyperplastic adrenal cortices. In these patients the gonads are ovaries but the external genitalia and secondary sexual characters may be more or less masculine in nature. A male pseudohermaphrodite is an individual whose gonads are testes but whose external genital organs and secondary sexual characters are more or less feminine. The genesis of this condition is not clear. Clinically, in either type of pseudohermaphroditism, the actual sex of the individual may be difficult or impossible to determine without surgical exploration. However, if further studies can substantiate a few reports, the determination of the 17-ketosteroid excretion may prove to be a very helpful diagnostic aid in the differentiation of these two conditions. Total excretion of 17-ketosteroids of 10 mg. and 5 mg. by 2 male pseudohermaphrodites, aged 15 and 26 years, were reported by Herweg *et al.* (123). Hain and Schofield (109) reported excretion of 13.3 and 7.4 mg. by a similar patient, 14 years of age. Thus, while the male pseudohermaphrodite appears to excrete normal amounts of 17-ketosteroids, the female pseudohermaphrodite excretes excessive amounts (see table 12).

True Hermaphroditism. A true hermaphrodite is an individual with both male and female gonads. The condition is rare. Weed *et al.* (262) reported a total output

of 17-ketosteroids of 7.6 and 7.5 mg/24 hours in a true hermaphrodite 36 years of age.

'Constitutional' Sexual Precocity. The terms 'natural,' 'idiopathic' and 'constitutional' have been applied to describe precocious sexual and somatic development in boys or girls for which no demonstrable pathologic cause has yet been found. In these, gross adrenal cortical, gonadal or cerebral lesions have not been demonstrated. A premature awakening of some of the natural processes of maturation appears likely. The sexual precocity adheres to the genetic sex of the child. Although these cases are not frequently reported, perhaps because of the undramatic pathologic and hormonal findings, it is likely that this type of precocity is the commonest of all types, in girls (186) as well as in boys (228). In only a few cases has the 17-ketosteroid excretion been determined, and in these the increase in output has been

TABLE 8. MYXEDEMA

REFERENCE	NO. OF CASES	17-KETOSTEROIDS, MG/DAY	REMARKS
		Spontaneous Myxedema	
(80)	6	1.2–3.3	5 females, 1 male
(90)	4	0.6–1.6	All females
(91)	3	0.0–1.7	All females
(92)	8	0.0–2.3	All females
		Secondary Myxedema	
(80)	4	2.5–6.7	3 females, 1 male (17-ks., 6.7)
(92)	5	1.7–5.8	All females

slight in amount. Nathanson and Aub (183) reported studies on 8 children (2 boys and 6 girls), 4 to 11 years of age, with abnormally early adolescence. In 2 the accelerated development was only slight. Excretion of 17-ketosteroids ranged from 3.5 to 14.9 mg/24 hours; this range was comparable to those obtained in normal individuals at or near puberty. The amounts excreted were more directly related to the physical and skeletal maturity of the patients than to their chronological age. Similar slight increases in excretion of 17-ketosteroids were found by Hain (108) in 5 girls and 2 boys and by Talbot *et al.* (241) in 4 girls and one boy. Apparently in this syndrome of precocious development the excretion of 17-ketosteroids never exceeds the normal adult range.

Sexual Precocity Secondary to Cerebral Lesions. Precocious development may be associated with tumors or inflammation in the region of the third ventricle or hypothalamus. We are not aware of any specific data on the excretion of 17-ketosteroids by members of this group. Since the clinical syndrome is like that of the con-

stitutional group and since the locus of the development of these abnormalities is undoubtedly similar, it would not be surprising if the 17-ketosteroid excretion were likewise similar.

Turner's Syndrome. A syndrome in women characterized by infantilism of the genital and secondary sexual organs, shortness of stature, primary amenorrhea, congenital webbing of the neck and cubitus valgus was described by Turner (253) in 1938. Also, other congenital anomalies, osteoporosis or delayed epiphyseal closure, have been described in some other cases with this general syndrome. Varney *et al.* (255) reported an excess of gonadotrophin in the urine in certain women with short stature and retarded sexual development. Albright, Smith and Fraser (3) reported that the excretion of 17-ketosteroids in 11 of their patients with this syndrome was depressed to 2 to 5 mg/24 hours (their normal range: 6 to 18 mg.). These values are higher than those seen in anterior pituitary insufficiency. Similar moderate depressions in the output of 17-ketosteroids associated with elevated amounts of urinary gonadotrophins have been reported for most cases of this syndrome by Schneider and McCullagh (225), by Dorff *et al.* (66), by del Castillo *et al.* (35) and by Greenblatt and Nieburgs (105). The significance and genesis of the lowered 17-ketosteroid excretion are unknown. Also, the syndrome, as yet, is not sharply delineated since the congenital developmental anomalies are not apparent in many of the cases, and occasionally the urinary hormonal pattern is not that which has been noted.

EFFECT OF STRESS

Attention has been directed by Selye (230) to the effect of stress on adrenal cortical activity in his descriptions of the 'alarm reaction' and the 'adaptation syndrome.' It is beyond the scope of this review to discuss the adaptation syndrome in detail. The effect of stress on the urinary 17-ketosteroids will be emphasized. Suffice it to state that there is some indirect evidence of an increase of adrenal cortical activity in response to the stimulus of stress (76, 203, 237, 250, 260, 263). In view of this evidence of increased cortical activity the effect of stress on the urinary 17-ketosteroids is somewhat paradoxical. An increase in adrenal cortical activity would be expected to result in an increased excretion of 17-ketosteroids. In general, the immediate effect of the stress of medical or surgical injury in previously normal individuals is to increase the amount of urinary 17-ketosteroids for one to 3 days. However, after this initial, brief rise the amounts excreted diminish to below control values and finally rise to normal when complete convalescence is achieved. Nevertheless, even while the 17-ketosteroid excretion is depressed there is indirect evidence of increased adrenal cortical activity. This evidence, though still in need of further study and interpretation, consists of an increased excretion of glycogenic corticosteroids and certain physiologic changes which are thought to be due to an increased production of such steroids. This apparent dissociation of functions of the adrenal cortex has not received an adequate explanation. It has been tacitly assumed that a diminished excretion of 17-ketosteroids is evidence of a decrease of some phase of cortical function. Apparently little consideration has been given to the possibility that diminished excretion may result from a change in the metabolic disposal of the 17-ketosteroids or their precursors, or both, although Cope *et al.* (41) have pointed out this possibility.

Albright (2) has elaborated a theory to correlate these divergent manifestations of adrenal cortical activity. This theory assumes a dissociation of adrenal cortical function associated with the urinary 17-ketosteroids and that function associated with the urinary glycogenic corticosteroids.

Surgical Operations. A major surgical operation constitutes a severe stress which is gradually relieved as convalescence progresses. Forbes *et al.* (85) found, in the case of normal men, that within 24 to 48 hours after an operation the excretion of 17-ketosteroids rose well above the upper limit of normal. Usually the peak value was found in the first specimen voided after operation but sometimes it did not appear until later. The amount then fell rapidly to a low point on the fourth or fifth day after operation. The return to normal occupied a variable length of time, with an average of 10 days. The response of normal women to stress was essentially the same as that of men but the peak was less pronounced or absent. The urinary 17-

TABLE 9. VIRILIZING OVARIAN TUMORS

REFERENCE	NO. OF CASES	AGE, YR.	17-KETOSTEROIDS, MG/DAY	REMARKS
(212)		16	1.86	Arrhenoblastoma
(90)	102	30	6.9 (av.)	Arrhenoblastoma
(197)[1]		24	4.0	Typical arrhenoblastoma
(137)	2	26	56, 36 (p.o.) 11	Arrhenoblastoma of intermediate cell type
(1)		22	40.0 (per liter)	No histologic data
(107)		12	116.0	Virilizing ovarian tumor. No clinical or pathologic data
(261)	87	31	158.0	No pathologic data
(140)		16	54.6 (p.o.) 2.6	Adrenal-like ovarian tumor
(197)[2]		44	23.8	Diffuse adrenal-like cells
(197)		50	12, 17 (p.o.) 1.8–8.6	Diffuse, nonlipoid containing adrenal-like tumor
(274)		65	43.0	Adrenal rest tumor of the ovary

[1] Mathiesen, cited by Pederson. [2] Luft, cited by Pederson.

ketosteroid levels after operations on the chronically ill or debilitated individuals of either sex were subnormal and the response to injury was small or absent. The general pattern of response described by Forbes *et al.* was seen frequently by Stevenson *et al.* (234), by Tompsett and Oastler (251), and by Cope *et al.* (41).

Stress of Daily Life. Pincus (203) interpreted the diurnal variations in 17-ketosteroid excretion as indications of variations in cortical activity in response to stress of daily life. It was found that a decreased amount of urinary 17-ketosteroids was associated with sleep whether at night or in the daytime. On waking at any time there was a prompt rise which was considered to indicate that waking involves a notable stress in terms of adrenocortical function. It was found also that a period of insomnia was marked by a rise in excretion of 17-ketosteroids, indicating a possible stress in staying awake. However, Tyler *et al.* (254) reported that prolonged wakefulness (112 hours) did not affect the amount of 17-ketosteroids excreted by young men. Amytal appeared to depress slightly the amounts excreted.

It has already been pointed out that a similar diurnal rhythm in water and

mineral exchange has been demonstrated by Manchester (158) and others. These observations deserve mention. Manchester, in an excellent study, showed that "the negative water balance, and urinary sodium, potassium and chloride output reached a maximum in the morning, declined in the afternoon and fell off sharply at night; water balance becoming positive and sodium, potassium and chloride excretion reaching a minimum in the early morning hours, coincident with the period of greatest water retention." He also showed that the rhythms of sodium and of potassium excretion could be reversed by desiccation and by fasting, respectively, and that physical exercise exerted little if any effect on the fluctuations noted. The fundamental cause of these fluctuations in renal clearance of water and electrolytes is not known. It would be impossible to conclude on the basis of excretion of 17-ketosteroids alone, without actual renal clearance studies on the steroids, that the increments in excretion of steroids which occur during waking hours are due solely to increases in adrenal cortical activity. However, the increments in excretion of steroids observed by Pincus were considerably greater than would be anticipated from alteration in renal clearance of these substances.

Exercise and Fatigue. Pincus and Hoagland (204), in an extensive study of the stress of flying, found an increase in diuresis and excretion of 17-ketosteroids which was directly proportional to the percentage of flying time and which was further augmented by increasing altitude. The excess excretion of 17-ketosteroids was correlated with good performance and resistance to fatigue.

Starvation. The excretion of 17-ketosteroids of 10 young men maintained on a diet which resulted in a loss of 24 per cent of their body weight during a 6-month period was reduced 30 per cent on the average (176). Young men who were trained to hard physical work and continued to work while fasting decreased their 17-ketosteroid output to 31 per cent of the control value by the fourth day of the regime. Landau *et al.* (148) found that 4 days of total starvation decreased the excretion of 17-ketosteroids 50 per cent in the case of 3 normal men and one obese woman. The values returned to normal about a week after feeding. However, a diet low in protein but only moderately restricted in calories failed to alter significantly the 17-ketosteroid excretion in spite of the fact that the subjects were in as great a negative nitrogen balance as were the starved individuals. It was concluded that inanition is one factor that will lower the urinary 17-ketosteroids in disease although there was no correlation between excretion of nitrogen and of 17-ketosteroids. Further, Landau *et al.* reached the conclusion that without more precise information concerning the source and metabolic pathways of the precursors of the urinary 17-ketosteroids, it was not possible to read a direct or simple interpretation of the secretory activity of the adrenal cortex from variations in the 17-ketosteroid excretion.

Anorexia Nervosa. Anorexia nervosa is discussed as a disease entity, but it is a condition resulting from prolonged inanition and, therefore, the subject is related to the discussion of the preceding section. However, in anorexia nervosa there is often evidence of partial pituitary failure, or, at least the two conditions resemble one another physiologically and clinically. In any event the failures in endocrine function in anorexia nervosa are reversed by adequate nutrition. Experimentally, inanition leads to lowered gonadotrophic and possibly adrenocorticotrophic activ-

ity of the anterior pituitary. In cases of anorexia nervosa very low values for urinary 17-ketosteroids have been reported, although some values within the normal range have also been obtained (table 14). The value of 14.7 mg. reported by Fraser and Smith (91) indicated that, in their case, there was no significant failure of the adrenal cortex to elaborate steroids. In the cases studied by Berkman, Carryer and Mason (12), in which the patients were women, half of the values were within the range found in cases of Addison's disease. Only 5 values were within the normal range and those were at the lower limit of normal. Except in one case, the excretions of 17-ketosteroids by the men were at the lower limit of normal or just below that limit. It is believed that these low levels of 17-ketosteroids indicate diminished adrenal cortical activity resulting from partial pituitary insufficiency although it may be that inanition affects the metabolism of steroids rather than their production.

Stress and Psychosis. In a study of the adrenal cortical response of psychotic men, Pincus (203) observed that these men did not respond to stress, such as exposure to cold and to heat or exposure to mildly anoxic conditions, with an increased excretion of 17-ketosteroids as did normal men. Examinations for other evidence of adrenocortical response indicated that psychotic men, on the whole, failed to exhibit

TABLE 10. 17-KETOSTEROIDS IN ADDISON'S DISEASE

	MEN		WOMEN	
REFERENCE	No.	17-KETOSTEROIDS, mg/day	No.	17-KETOSTEROIDS, mg/day
(29)	3	3.5–7.5	4	3.1–7.5
(90)	3	2.1–3.5	5	0.5 (all cases)
(92)	3	0.0–3.0	2	1.0 (av.)
(257)	1	2.3–3.8	3	1.9–6.8

a typical response to stress. That this failure was not due to an inability of the adrenal cortex to respond to the stimulus of stress was shown by a normal response to stimulation of the cortex with adrenocorticotrophin. The defect appears to be in the adrenocorticotrophic function of the pituitary.

MALIGNANT LESIONS AND 17-KETOSTEROIDS

The relation of the presence of malignant tumors in man to the excretion of 17-ketosteroids has received considerable study because of various suggestions as to a possible relationship of steroids to carcinogenesis. Pearlman (195) found that cancerous perons excreted significantly less 17-ketosteroids than normal persons and that the normal sex difference in excretion is abolished in cancerous persons. However, it has already been noted that a great variety of unrelated acute and chronic disorders may depress the total quantity of 17-ketosteroids in urine. Unfortunately, in none of these latter states has the qualitative composition of the urinary steroids been examined in any detail although such studies have been made for normal individuals. To demonstrate that the quantity or pattern of the urinary steroids of cancerous persons differs from that of normal individuals does not permit the conclusion that the observed difference is due to the influence of the cancerous process per se or to an abnormal steroid metabolism which might be a prerequisite for the development of

cancer. To approach this conclusion would involve a comparison with individuals not suffering from cancer but who are in a comparable physiologic state of health. Thus far, most of the studies of the urinary steroids of cancerous patients suffer from lack of this type of control data. In addition, when abnormal excretion of steroids is observed, present knowledge does not allow distinction between an effect produced by altered adrenal cortical activity and one produced by altered metabolism of precursor hormones.

Prostatic Cancer. Men with cancer of the prostate excrete amounts of 17-ketosteroids that are still within the range excreted by normal men of comparable ages (89, 221, 226). That orchectomy causes an initial fall and a subsequent rise in 17-ketosteroid excretion has already been mentioned. According to Satterthwaite *et al.* (221), the clinical improvement immediately after castration is correlated to some extent with the percentage decrease in excretion of 17-ketosteroids.

Salter, Humm and Goetsch (216) studied the relation between the urinary 17-ketosteroids and estrogens in prostatic disease. The ratio of 'estroids' (in μg.) to 17-ketosteroids (in mg.) was less than 1.0 in healthy young men and 2 to 10 in normal ovulating women. The ratio for men with prostatic carcinoma or hypertrophy was frequently in the female range and occasionally more than 10.

Mammary and Gastric Cancer. According to Taylor *et al.* (247) and Nathanson (182), the total excretion of 17-ketosteroids in cases of cancer of the breast does not differ from that in persons of comparable physiologic status but without cancer. By means of isolation methods Hill and Longwell (124) failed to show any steroid uniquely related to malignant disease. However, the more detailed studies of Dobriner and his associates (59–61, 65, 153) have shown qualitative changes in the 17-ketosteroid pattern which may be correlated with malignancy, not only in mammary and gastric but also in prostatic cancer. In cases of gastric cancer Dobriner *et al.* (61) found that androsterone and etiocholanolone were present in small amounts. The pattern was very similar to that observed in cases of prostatic cancer.

11-Hydroxyetiocholanolone (isolated as the product of dehydration, $\Delta^{9:11}$-etiocholanolone) (62) was found in the urine of many patients suffering from these malignant tumors. Although this compound has not been detected in the urine of normal young men and women (153), its presence cannot be considered to be entirely specific for the presence of a malignant lesion since it has been found by these investigators in the urine of some patients with essential hypertension and Cushing's syndrome as well as in the urine of a woman, aged 76 years, and a man, aged 75 years, both of whom were in good health. The presence of 11-hydroxyetiocholanolone may still be significant in malignancy since Dobriner (59) isolated it from the urine of a woman in whom cancer of the breast developed several months subsequent to collection of the urine. However, it is not clear why this compound should be any more significant than 11-hydroxyandrosterone or 11-ketoetiocholanolone with respect to production or metabolism of 11-oxygenated adrenal cortical hormones. These two compounds, which are so similar in chemical structure to 11-hydroxyetiocholanolone, have been isolated from the urine of normal individuals (152, 163).

Leukemia. Levin (151) observed a markedly reduced excretion of 17-ketosteroids by 3 men and 2 women with lymphatic leukemia. The excretion of 17-ketosteroids was not depressed in 3 cases of myelogenous leukemia.

TABLE 11. URINARY 17-KETOSTEROIDS IN CASES OF ADRENAL CORTICAL TUMOR

NO.	SEX	AGE, YR.	TOTAL 17-KETOSTEROIDS MG/DAY	β FRACTION, APPROXIMATE PER CENT OF TOTAL	REFERENCE
Cases Associated Primarily with Masculinization					
1	F	5.5	19–22	1	(271)
2	F	41	76, 78		case 4 (32)
3	F	2	75	73	case 2 (135)
4	F	31	78.5	79	case 1 (135)
5	M	10	84		case 5 (107)
6	F	1	88.8	87	(150)
7	F	26	107		case 3 (32)
8	F		133	50	case 15a (240)
9	F	3	160	65	case 9 (242)
10	F	3.5	176 (av.)		case 40 (90)
11	F	4	320, 166		case 3 (107)
12	F	25	367	30	case K (9)
13	F.	45	857		case 8 (81)
Cases Associated Primarily with Cushing's Syndrome					
14	F	36	19.9[1]		case 35 (90)
15	F	40	30	7.5	case 16 (141)
16	M	25	40–64	20	case 1 (43)
17	F	21	45		case 1 (107)
18	F	38	54, 52		case 5 (32)
19	F	56	72		case 34 (90)
20	F	56	74	22	case 14 (240)
21	M	46	92.7		(270)
22	F	25	131		case 5 (81)
23	F(?)		170		case 3 (193)
24	F	6	126–288	65	case 2 (43)
25	F	34	270		case 4 (193)
26	F	53	800 (av.)	69	case 14 (141)
Cases Associated with Various Degrees of Masculinization and Cushing's Syndrome, or Without Classification					
27	M	1.25	2.8		case 1 (81)
28	F	1.25	3.0		case 2 (81)
29	M[2]	5	5.1		(271)
30	M	39	4.8[3]		case 17 (141)
31	F	3.5	5.9		case 2 (107)
32	F	61	14.5–20[4]		case 1 (32)
33	M	5	27		case 2 (193)
34	M	1	28 (per liter)		case 1 (193)
35			45		(95)
36	F	63	45		case 3 (81)
37	F	16	54		case 4 (81)
38	M	32	68	48	case 18 (141)
39	F	54	83		case 66 (261)
40	M	43	86	57	case 3 (135)
41	F	40	126		case 99 (261)
42	F	13	166	50	case 1 (242)
43	F	3.5	170		(81)
44	F	25	215		(4)
45	F	21	240		case 7 (81)
46	F	49	269	Isolated DHA[5]	case 79 (261)
47			325		(95)
48	M	7	275–420		(127)
49	F	42	690	Isolated DHA[5]	case 75 (261)
50	F	55	1005	77	case 15 (141)

[1] Urine stored 5 years before assay.

[2] 'Feminized' in that breasts were enlarged. No virilism except for prostatic enlargement. First case of gynecomastia due to adrenal tumor reported in a boy before puberty.

[3] Probably a nonfunctioning adrenal cortical tumor.

[4] Patient 'extremely ill' at time of urine collection.

[5] DHA = Dehydroisoandrosterone

17-KETOSTEROID EXCRETION IN MISCELLANEOUS CONDITIONS

Mental Deficiencies. Benda and Bixby (11) found that familial morons of both sexes and patients with various types of brain injury excreted normal amounts of 17-ketosteroids. The amounts excreted by mongoloid children and females of adult age varied from subnormal to high normal levels, while mongoloid males of adult age tended to have low normal levels. There was no consistent tendency to either hypoexcretion or hyperexcretion.

Electroshock Therapy in Agitated Depression. Hoagland *et al.* (130) found that in the case of normal individuals the ratio of the amount of 17-ketosteroids excreted per hour in the morning to that excreted during sleep is about 1.60. In the case of women suffering from agitated depressions the average of this ratio was 1.03. This ratio was increased for most, but not all, patients who received electroshock therapy, the average ratio becoming 1.32. There was no evident correlation between psychiatric improvement and increase in the ratio.

Prefrontal Lobotomy. Reitman (213) investigated the effect of bilateral prefrontal lobotomy on the excretion of 17-ketosteroids by 12 adult females. Three to 4 months after the operation there appeared to be a statistically significant decrease in the amount of 17-ketosteroids in the urine. On the other hand, Hemphill *et al.* (120) found that the 17-ketosteroids excreted by 6 men subjected to the same type of operation 3 months previously increased slightly. Further investigations are necessary to clarify the reasons for these divergent results.

Myotonia Dystrophica. This disease is a particular type of familial muscular dystrophy which is associated with pathologic lesions, mainly atrophic in nature, of the gonads, adrenal cortices and anterior pituitary. In keeping with testicular and adrenal atrophy, Benda and Bixby (11) found the 17-ketosteroid excretion was low in this condition, 4.8 to 8.0 mg. per day in the case of 4 men, 31 to 36 years of age, and 2.8 mg. per day in the case of a woman, 39 years of age. Excretion rates below the range of normal have been observed by one of us (78) in 2 adult males with this condition.

Hypertension. In cases of hypertension the average excretion of 17-ketosteroids is generally but not always lower than in normotensive individuals. No specific alteration in output of 17-ketosteroids can be considered to exist other than that seen in ill individuals. Bruger *et al.* (17) found no difference in incidence of hyperplastic or adenomatous changes in the adrenal cortex of hypertensive as compared with normotensive individuals. In more than 7000 consecutive necropsies Commons and Calloway (39) were unable to detect any significant difference in the incidence of hypertensive cardiovascular disease, cardiac enlargement, diabetes mellitus or gonadal changes in patients with adenomas of the cortex as compared to that in patients without such changes. However, Selye (229) felt that her results indicated an increase in some phase of adrenal cortical function in some cases of essential hypertension in which there was an "increased serum Na to Cl ratio" and an increase in the excretion of corticosteroids. In one case of malignant hypertension Tompsett and Oastler (251) were unable to discover any significant change in the excretion of 17-ketosteroids or corticosteroids during a severe attack of hypertension.

Mastitis. In 4 cases of chronic mastitis in which the patients were women, aged 27 to 50 years, Tompsett and Oastler (251) reported consistently low values of 3.5 to 4.6 mg. per day. Nathanson (181) found that adolescent mastitis in males was associated in many instances with atypical ratios of urinary 17-ketosteroids to urinary estrogens. The condition was considered to be more closely related to the ratio of urinary 17-ketosteroids to urinary estrogens than to the actual value of either urinary principle.

Gynecomastia of Malnutrition. Determinations of the 17-ketosteroids were made

by Salter *et al.* (218) in 27 cases of gynecomastia of nutritional origin one to 38 months after onset of the gynecomastia and when the subjects (military personnel) had returned to a normal weight. In addition to gynecomastia these patients showed testicular atrophy, oligospermia and decreased potency. The concentrations of 17-ketosteroids varied from 4.1 to 15.0 with an average of 10.3 mg. per day as compared with normal control values of 3.1 to 23.0, average 14.2 mg. per day. Urinary estrogens were in the low normal range and the ratio estrogens to 17-ketosteroids was normal. A heterogeneous group of civilian patients with gynecomastia showed essentially the same pattern of urinary steroids and a normal estrogen to 17-ketosteroid ratio. These

TABLE 12. EXCRETION OF 17-KETOSTEROIDS IN CASES OF PREPUBERTAL VIRILISM WITH PROVEN ADRENAL CORTICAL HYPERPLASIA AND IN CASES OF FEMALE PSEUDOHERMAPHRODITISM IN WHICH CORTICAL HYPERPLASIA IS HIGHLY PROBABLE

CASE	AGE, YR.	17-KETOSTEROIDS, MG/DAY	β FRACTION, PER CENT OF TOTAL	REFERENCE	CASE	AGE, YR.	17-KETOSTEROIDS, MG/DAY	FRACTION, PER CENT OF TOTAL	REFERENCE
51	3	13.5		case 9 (81)	64[1]	11	69		case 1[1] (98)
52	4	37		case 2 (98)	67	12	32		case Mot. (16)
53	5	37		case 10 (81)	68	13	23.4	3	case 11 (240)
54	5	6		case HA (16)	69	13	64		case 6 (193)
55	5	19.2	17	case 4 (240)	70	16	43		case Sim. (16)
56	6	12.4		case BA (16)	71	17	35		case 3 (193)
57	7	37		case Eg (16)	72	17	54		case Her. (16)
58	7	34, 37		case 4 (193)	73	18	56		case 2 (193)
59	8	11.7	0	case 8 (240)	74	18	54		case 5 (193)
60	8	23.4	7	case 10 (240)	75	19	75.2		case 11 (81)
61	8	17.0	23	case 1 (242)	75	21	123[2]	20	case 11[2] (141)
62	9	52		case 1 (193)	76	21	35–74		case 12 (107)
63	9	50		case 7 (193)	77	22	75		case Ron. (16)
64	10	30		case 1[1] (98)	78	23	60	9	case 4 (242)
65	10	31	14	case 19 (141)	79	41	102 (av.)		(78)[3]
66	11	29	0	case 2 (242)					

[1] Observations made one year after observations in *case 64* listed above. [2] Observations 2 years later. [3] New Haven Hospital *Case B93002.*

results distinguish this group of patients from those having gynecomastia associated with hepatic cirrhosis or acute hepatitis. Since the patients with gynecomastia of nutritional origin were not studied at the inception of the disorder, it is still possible, however, that a temporary derangement of liver function resulting from the malnutrition may have played a prominent part in the pathogenesis of the gynecomastia. Such an interpretation is indirectly supported by the work of Biskind and Biskind (13) who found that deficiency of vitamin B-complex impaired the ability of the liver, in rats, to inactivate estrogens whereas the ability to inactivate testosterone remained unimpaired.

Hepatic Disease. Impairment of liver function to any marked degree is associated with diminished amounts of urinary 17-ketosteroids. In acute infectious hepatitis

(100) and cirrhosis (102, 103) the amounts excreted are often very small. Not uncommonly the latter disease is accompanied by gynecomastia, testicular atrophy, decreased total body hair and loss of libido. It is not obvious why liver disease specifically should decrease the excretion of 17-ketosteroids. The excretion of estrogens tends to increase under these circumstances. The gynecomastia has been attributed to the presence of an increased amount of estrogen, much of it unconjugated, in the face of a diminished amount of androgens. In acute infectious hepatitis Gilder and Hoagland (100) found an increase in the excretion of estrogens and a marked to moderate decrease in the excretion of 17-ketosteroids. Gynecomastia was not mentioned in their report. The excretion of 17-ketosteroids returned to normal levels considerably in advance of the estrogens and of clinical recovery.

Ankylosing Spondylarthritis and Rheumatoid Arthritis. Davison *et al.* (47) determined that 13 male patients suffering from ankylosing spondylarthritis excreted, on the average, 27.3 mg. per day of 17-ketosteroids as compared with their normal average of 14 mg. per day. Treatment with roentgen rays (48) caused temporary reduction in the amount of urinary 17-ketosteroids and induced remissions of the

TABLE 13. EXCRETION OF 17-KETOSTEROIDS IN CASES OF POSTPUBERTAL VIRILISM WITHOUT ADRENAL TUMOR

NO. OF CASES	AGE, YR.	URINARY 17-KETOSTEROIDS, MG/DAY Range	Mean	REFERENCE
7	21–44	10.0–30	16.5	(32)
67	18–34	6.4–33.4[1]	19.9	(193)
20	19–36	5.9–37	22.0	'Adrenal virilism' (261)
13	20–36	3.4–33.0	16.0	'Hirsutism' (261)
4	16–54	23.1–37.0	28.0	'Simple hirsutism' (257)
21		1.0–29.0		(95)

[1] Less than half of these patients excreted amounts clearly in excess of normal.

disease but the excretion of 17-ketosteroids soon increased again to high levels even though the disease remained in abeyance. These authors (47) also determined that the average excretion (12.8 mg.) of 11 women suffering from rheumatoid arthritis was slightly higher than the average value (10 mg. per day) for normal women.

These results are of considerable interest in view of the recent discovery that 17-hydroxy-11-dehydrocorticosterone (cortisone) causes remissions in rheumatoid arthritis and other rheumatic diseases. Some relation may exist between adrenal cortical function and the state of these disorders, but further interpretation of the data is not possible.

Gouty Arthritis. Consistently low excretions of 17-ketosteroids by patients (10 males and one female) with gout recently have been reported by Wolfson *et al.* (277). The range of the amounts excreted was 0.67 to 6.6 mg. per day, with an average of 3.0 mg. Six of the 11 values were below 2.5 mg. per day. The low output was present during asymptomatic intervals as well as during acute attacks. Gonadal function appeared to be intact and there was no evidence of pituitary, thyroid or adrenal cortical failure in these patients. If these data can be further substantiated, a dysfunction in precursor hormone production or in the metabolic disposal of steroids is a possible cause of these findings.

Renal Insufficiency. Huis in't Veld and Querido (134) found a value of 10.7 mg. per day for the urinary 17-ketosteroids in a case of uncomplicated renal insufficiency. Of this total, only 0.3 mg. was in the free form. It was concluded that the kidney is not required for the conjugation of 17-ketosteroids.

Benign Hypertrophy of the Prostate Gland. Miller (172) isolated androsterone and 3-chlorodehydroandrosterone from the urine of men with benign hypertrophy of the prostate gland. The amount of androsterone isolated (0.02–0.04 mg. per liter) was considerably less than that obtained by Callow and Callow (28) from the urine of normal men (1.6 mg. per liter), but if the uncertainties of isolated methods are considered, the difference may not be significant. Also, the amount (0.09 mg. per liter) of dehydroisoandrosterone (assuming this substance was the precursor of 3-chlorodehydroandrosterone) probably was not significantly different from the amount isolated (0.2 mg. per liter) from the urine of normal men. Satterthwaite *et al.* (221) found that the mean level of 17-ketosteroids in 10 cases of benign prostatic hypertrophy did not differ significantly from the mean level in cases of prostatic carcinoma.

Migraine Headache. Torda and Wolff (252) observed increased amounts of 17-ketosteroids in the urine during attacks of migraine headache. This increase may have resulted from the effect of the stress of headache.

17-KETOSTEROIDS IN VARIOUS ANIMALS

17-Ketosteroids have been found in the urine of various species. It is clear from these studies that steroid output varies considerably in different species of animals.

Marker (149, 160) isolated androsterone and dehydroisoandrosterone from the urine of bulls and steers. Androsterone also was isolated from the urine of pregnant cows (160) and indications of the presence of dehydroisoandrosterone were obtained.

Duyvené de Wit (75) found 375 μg. of 17-ketosteroids per liter of ovarian follicular fluid of the pig, cow and human. Green and Winters (104) observed that the 17-ketosteroid content of boar's urine varied with the age of the animal and the line of breeding. There was an association between the amount of 17-ketosteroids excreted and the sexual behavior of the boars.

The urine of 2 pregnant ewes (269) carrying ram lambs contained amounts of 17-ketosteroids of the same order as those recorded for normal human females. According to Paschkis *et al.* (191) normal dogs excreted only from 0.5 to 1.5 mg. of 17-ketosteroids per day; there was no difference between the sexes. Unlike human beings, the administration of testosterone propionate to normal male and castrated dogs did not lead to an increased urinary output of 17-ketosteroids or androgens; rather an increased excretion of estrogens occurred.

Dorfman and his associates (69) found 1.6 to 2.0 mg. of 17-ketosteroids per day in the urine of adult Rhesus monkeys. Even after removal of the gonads and adrenal glands, both male and female monkeys continued to excrete about one-third as much 17-ketosteroids and androgenic activity as before operation. These results afford evidence that the monkey has some extragonadal and extra-adrenal source of 17-ketosteroids.

Karnofsky *et al.* (138) found that mice of strains having a high (C_3H) and low (C_{57}) incidence of mammary cancer excreted 0.46 to 1.93 mg. of 17-ketosteroids per

month in the urine. There was no appreciable difference between the strains. Samuels *et al.* (219) observed a fecal excretion of 17-ketosteroids by mice of various strains, most of which were in an unconjugated form. Those mice which had the milk agent that causes mammary tumors excreted less 17-ketosteroids in the feces than those which did not have the milk agent.

Adult female rabbits of the New Zealand strain were studied by DeKoning *et al.* (52, 53). Intact and ovariectomized animals excreted 1.5 to 4.0 of 17-ketosteroids/48 hours and there was no statistical difference between the two groups. There was no correlation between urine volume and amount of 17-ketosteroids. Oral administration of sodium estrone sulfate to the intact and ovariectomized rabbits had no effect on the excretion of 17-ketosteroids over a period of 192 hours.

Kimeldorf (142) also studied the excretion of 17-ketosteroids by rabbits and found that the average level of excretion by the normal male rabbit was 2.2 mg/48 hours. No relation to volume of urine was noted. Destruction of the adrenal cortex resulted in a significant lowering of the 17-ketosteroid output. Castration (143) and experimental cryptorchidism depressed the excretion of 17-ketosteroids by 41 per

TABLE 14. ANOREXIA NERVOSA

REFERENCE	NO. OF CASES	AGE, YR.	17-KETOSTEROIDS, MG/DAY	METHOD[1]
(90)	5	20–31	2.3–7.2	C
(91)	4	20–27	2.7–14.7	
(257)	3	24–37	3.5–7.2	HK
(12)	21 (F)	15–43	0.3–5.8	C
	4 (M)	33–44	(Av. 2.4)	
			4.6–11.4	
			(Av. 6.6)	

[1] C = Callow, Callow and Emmens (30) HK = Holtorff and Koch (132).

cent. The amount of urinary 17-ketosteroids was not increased by administration of a polyvalent pituitary extract (Polyansyn) to normal animals but was increased by administration of testosterone. These studies would imply the adrenal cortical and testicular origin of the urinary 17-ketosteroids in rabbits.

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SURVIVAL AND REVIVAL OF NERVOUS TISSUES AFTER ARREST OF CIRCULATION

C. HEYMANS

From the Department of Pharmacology and Therapeutics, University of Ghent

GHENT, BELGIUM

THE PROBLEM OF SURVIVAL AND REVIVAL of different parts of the nervous system, after an arrest of the circulation of the blood during a given length of time, is directly connected with the more general problem of life and death. Speaking of an organism as a whole, all biologists will agree that death is a condition in which some irreversible damages have occurred in some fundamental organ, tissue and physiological function of the body—damages which make normal activity and life of the organism as a whole impossible. One of the main factors inducing such irreversible damages is the arrest of the heart and of the circulation of the blood, which provokes anoxia and asphyxia of all cells, tissues and organs. This pathological condition may induce, after a given length of time, irreversible damages which make normal activity and life impossible. The question arises: What is the resistance of the different tissues and physiological functions of the organism to the interruption of blood supply and to anoxia?

It is a well known fact that the muscles, the intestines and even the heart are very resistant to the interruption of blood supply and anoxia. These tissues and organs may indeed be revived and resume their activities even days after the blood supply has been interrupted. But not all cells, tissues, organs and physiological functions are so resistant to the deprival of blood supply and anoxia, and do not resume their activities, after a period of depression and apparent death, when the circulation and oxygen supply have been restored.

Different parts of the nervous system are much more sensitive to lack of oxygen and deprival of blood supply than are other tissues. Stenon (210) and later Legallois (156) were the first to perform experimental investigations on this fundamental problem. Since these preliminary observations, much research has been devoted to the same problem.

We intend to survey in this review the main experimental contributions to the effects of arrest of circulation, asphyxia and anoxia on the survival time and on the revival of different nerve tissues. Considerable information has also been reviewed in publications of Cannon and Burket (39), Gellhorn and Pollack (84), Van Liere (234), Hoff and Fulton (135), Sauzède (193) and Nicholson (173).

FUNCTIONS OF MEDULLA OBLONGATA AND BRAIN IN ARREST OF CIRCULATION

Anemia of the brain was produced experimentally by occlusion of the arterial supply as early as 1836 by Astley Cooper (45a) and since then by Pike, Guthrie and Stewart (180), Hill (128, 129), Crile and Dolley (49), Gildea and Cobb (94) and Petroff (179). Anemia of the brain by occlusion of arteries is only temporary and

incomplete because of important collateral circulation. Porta (183), Schiff (194), Kussmaul and Tenner (145), Strelkoff (218), Ljubomudroff (160) and others obtained full recovery of dogs after ligation of both carotid and vertebral arteries and showed that ligature of both common carotid arteries, vertebral and spinal arteries in dogs does not induce an anemia of the brain incompatible with normal functions of nerve centers. Indeed the animals manifest all the functions normally, although the finest processes of higher nervous activity may be disturbed. Andreyev (4) thus showed that after ligation of the arteries supplying the brain in dogs, the fundamental conditioned reflexes are weakened. Anemia of the brain was also produced by temporary arrest of the heart or by injection of substances causing thrombosis of the arteries (46, 166, 237). Ligature of the arteries to the brain in cats induces a more complete decrease of blood supply to the nerve centers (94), as the animals do not recover from this operation.

Brown-Séquard (33–36) performed the ligature of the aorta above the renal arteries and the carotid and vertebral arteries in dogs, rabbits and guinea pigs. He controlled the survival of the animals. Hill and Mayer (129, 172), Prus (185, 186), d'Halluin (54), Stewart *et al.* (103, 211–215), Batelli (12, 13) and Crile and Dolley (48, 49) have investigated the effect of complete anemia of the central nervous system, mainly in dogs. Gomez and Pike (96) also performed total anemia of the central nervous system in cats. Weinberger *et al.* (239) working with cats produced temporary anemia by occluding the pulmonary artery and examined the pathological changes in the cerebral cortex.

Although some interesting information was given, these different groups of experiments performing ligature of embolism of the brain vessels did not answer the questions concerned with the resistance of different nerve centers to complete anemia and the capacity of revival of the nerve centers after a certain duration of interruption of blood supply. These experiments were not suitable because of the different species of animals used and the existence and vicarious enlargement of the collateral vessels and development of new anastomosis. Therefore, experiments on total anemia and resuscitation of the isolated nerve centers had to be performed by perfusion of the isolated head.

Laborde and Gley (146–148) were the first investigators to try perfusion and revival of the isolated head. These experiments were performed on the head of man after decapitation. The results, however, were practically negative. Hayem and Barrier (104) perfused the isolated head of dogs with cow blood. The experimental conditions were not favorable and thus the observations were practically negative. In 1912, J. F. Heymans with his assistant de Somer (122) performed the first really successful experiments of perfusion, anemia and resuscitation of the isolated head of dogs, rabbits and cats. This somewhat modified technique has been used in a number of experimental investigations by J. F. and C. Heymans (122–124), C. Heymans and Ladon (120, 121), C. Heymans (110, 112), C. Heymans and Bouckaert, (113–115), C. Heymans, Bouckaert, Jourdan, Nowak and Farber (116), and Nowak and Samaan (177, 178). Pike, Guthrie and Stewart (103, 180, 181), Brukhnenko and Tchetchuline (37), Kabat and Dennis (138) and Kabat (137) also performed anemia and resuscitation of the brain by perfusion.

C. Heymans *et al.* (110, 113, 114, 116, 119–121) pointed out that their experiments demonstrated that the respiratory, cardioregulatory and vasomotor centers possessed great resistance to anemic anoxia and could still be revived in dogs after the circulation had been arrested completely for as long as 30 minutes. They stated, however, that certain centers were more sensitive to anemia. The palpebral and pupillary centers and the cortical centers were irreparably damaged and could not be revived if the circulation were arrested for more than 10 and 5 minutes, respectively. Table 1 shows the ability of centers to revive after complete interruption of blood supply (C. Heymans *et al.*, 116).

Sauzède (193) reported that in rabbits the respiratory center and some other nerve centers may be revived after the circulation has been arrested completely for as long as 47 minutes. These experimental observations thus showed how resistant some nerve centers are to complete interruption of blood supply. In 1866, Vulpian (237) pointed out that the bulbar centers are more resistant than the cortex to arrest of circulation. Batelli (12, 13), de Cyon (53) and Pike, Guthrie and Stewart (180) also observed that the circulatory and respiratory centers could be revived after 20 to 30 minutes of arrest of circulation. The palpebral reflex center may resist, in dogs and rabbits, up to 20 to 25 minutes of arrest of circulation (12, 13, 53).

In *in vitro* conditions, the oxygen consumption of the medulla was found to be definitely less than that of the cortex and basal ganglia (43, 47). It is well known from earlier observations of Haldane (106), Barcroft (9) *et al.* that the highest mental functions suffer first in conditions of anoxia. The same phenomenon was brought out more quantitatively by studies of McFarland (162) and Gellhorn and Kraine (82). Cortical sensory and motor functions are likewise altered in anoxia. Effects of anoxia on cortical processes have been investigated also by means of action potential, by the electroencephalogram, or, in animals, by leads taken directly from the exposed cortex. Sugar and Gerard (219, 220) studying the brain potentials in cats reported that after abrupt and complete anemia of the brain, it took 14 to 15 seconds for the disappearance of the potentials in the cerebral cortex, whereas it took 30 to 40 seconds to make them disappear from the region of the medulla. Orthostatic hypotension, occlusion of cerebral vessels, critical rise of cerebrospinal fluid pressure, and in some cases of hyperactive carotid sinus reflex inducing fall in blood pressure cause slow waves and then flattening of cortical activity (61, 69, 70, 167, 216, 217).

Hoagland (134) and Davis *et al.* (50, 51) observed that, with a decrease in oxygen tension, the frequency of the alpha waves decreased. Later on, slower waves with larger potentials (delta waves) appeared. When these had more or less completely replaced the alpha waves, consciousness was lost. The delta waves are, in general, interpreted as indicating synchronous discharges of a reduced number of brain cells.

Observations on non-narcotized animals show that in the course of anoxia, the cortical potentials gradually decrease in size and frequency and may disappear completely. On readmission of oxygen they recover in a very short time (24). Experiments also show that the sensitivity to anoxia is greatest in the cortex and less pronounced in subcortical structures (86, 90, 138). The reason for the lesser suscepti-

bility of the brain stem to anoxia seems to be partly due to its dependence on cortical inhibitory influences (87). Chauchard (41, 42) showed changes in motor cortical excitability at high altitude. Excitability of cerebral cortex is increased in moderate hypoxemia. This action is reversed by increasing the CO_2 pressure (143, 176).

Dusser de Barenne, Marshall, Nimes and Stones (56) reported a distinct differential effect of anoxia on normal and strychnine potentials of the cortex; whereas Noel (175) noted a simultaneous disappearance of convulsive and normal potentials during asphyxia. Gellhorn and Heymans (79) showed that anoxia abolishes convulsive potentials at a time when the normal potentials are practically unchanged. The sensitivity of the cortex to anoxia and anemia thus depends also on the condition of activity of the cortical centers.

It has been known for a long time that young animals are less susceptible to anoxia than adults. As early as 1725, Boyle (23) mentioned the resistance of kittens to asphyxia, and Paul Bert (18) called attention to the fact that newborn animals are more resistant to asphyxia than adults. Similar observations have been collected by a number of observers (6, 131, 188, 202). More recently Kabat *et al.* (137) showed

TABLE 1. ABILITY OF CENTERS AT VARIOUS LEVELS OF NERVOUS SYSTEM TO WITHSTAND COMPLETE ANOXIC ANEMIA

INTERRUPTION OF CENTRAL BLOOD SUPPLY UP TO	CORTICAL CENTERS	PALPEBRAL PUPILLARY CENTERS	CARDIO-REGULATORY CENTER	VASOMOTOR CENTER	RESPIRATORY CENTER
1– 5 min.	+	+	+	+	+
5–10 min.	–	+	+	+	+
10–15 min.	–	–	+	+	+
15–30 min.	–	–	+	+	+
30 min.	–	–	–	–	–

that very young dogs were found to be much more resistant to acute asphyxia than adult animals. The respiratory center in newborn animals continued to function 17 times as long as in adults, following complete arrest of blood flow to the brain. The corneal reflex disappeared in 10 seconds, and the respiratory center ceased functions within 20 to 30 seconds. If the arrest of circulation was not longer than 6 minutes, there was complete recovery of function, but interruption of blood flow for 8 minutes or longer produced permanent damages to the brain. Fazekas, Alexander and Himwich (65), and Glass *et al.* (95) observed higher resistance to anoxia in newborn rats, rabbits, dogs and guinea pigs than at any subsequent time.

Selle (201) found prolongation of gasping pattern of young anoxic animals by administration of carbohydrates and its abbreviation by insulin hypoglycemia. Himwich and Fazekas (133) pointed out that, in the adult dog brain, the caudal nucleus and the cerebral cortex have the highest metabolic needs, whereas the medulla and midbrain are highest in the week-old puppy. Essential corroboration of these facts has been given by Tyler and van Harreveld (226).

Some of the factors affecting resistance to anoxia in mice have been investigated by Hiestand and Miller (126), and by Kibrick and Goldfarb (140). Starvation, dehydration, food intake, and rate of ascent are significant variables in experiments

testing the response of animals to anoxia. High carbohydrate diet increases tolerance in man to anoxia (141). Gellhorn and Kessler (80, 81) have shown that low blood sugar increases the effects of oxygen lack on cortical activity. Increased metabolism also increases sensitivity to oxygen lack. Green, Butts and Mulholland (99) and Eckman *et al.* (59) also reported an increase in resistance to anoxia from a high carbohydrate diet. McFarland *et al.* (163), Riesen, Tahmisian and Mackenzie (189) described a lowered sensitivity to oxygen want from injection of glucose. Himwich and associates (132) showed that insulin in the newborn decreased the survival time in anoxia, while glucose increased the survival time. Injection of iodoacetate and fluoride cut down the survival time of the newborn. Gellhorn and Kessler (80, 81) found that the effect of anoxia on brain potentials is increased by insulin; this effect can be alleviated by inhalation of pure oxygen. Smith and Oster (204) found that insulin decreased resistance to anoxia. Leblond (155) reported that thyroidectomy increases the resistance to anoxia.

Charipper *et al.* (40) observed that rats whose diet was deficient in vitamin B, showed increased resistance to anoxia, an effect counteracted by thiamine. Thiourea and thiouracil also induced increased resistance of rats to anoxia, while estrogens were without effect. Thyroxin has the reverse effect. Thorn *et al.* (223) reported the same observations for adrenal cortical extract, but not for desoxycorticosterone, 17-hydroxycorticosterone or sodium succinate. Hiestand and Brodie (125) found increase of resistance of the respiratory center in the isolated rat head by vasopressine and glucose, while oxytocin had no effect. Snyder (205) observed that sodium pentobarbital increased the resistance of the respiratory center to asphyxia in newborn rabbits. Kalk and Brühl (139) found decreased resistance to anoxia in fever infections, exudative pleuritis, hyperthyroidism and fatigue, with increased resistance to anoxia in some liver diseases and allergies. Lawson (154) reported that sulfanilamide increased the tolerance of rabbits to oxygen lack. Heymans (111) showed that di-isopropylfluorophosphonate (DFP) increases from 30 minutes to 90 minutes the ability of recovery of the respiratory and circulatory centers of the isolated perfused head after interruption of blood flow.

Freedman and Himwich (72) observed that DFP does not increase the resistance of rats to acute anoxia. Welsh and Hyde (240) feel that resistance to anoxia is related to the acetylcholine content of the tissues. Gellhorn (74) has shown that mice cannot survive a low pO_2 as well at high as at room temperature. Miller (169) found that cooling of young animals increases their resistance to anoxia. The survival time of dogs and rabbits breathing mixtures low in oxygen is greatly shortened by administration of moniodoacetic acid (66, 67).

However, the marked difference in sensitivity to hypoxia of the medulla oblongata as compared with that of the cortex, and the influences of different factors on this resistance, is not only based on quantitative differences in the metabolic needs of these structures. The fact that the respiratory center may be noticeably stimulated in anoxia, when cortical activity is decidedly depressed (78), points out the fact that qualitative differences exist in the reactivity of the medulla and the supramedullary mechanisms (76). These differences are also based upon the fact that the chemoreceptors of the cardio-aortic and carotid sinus regions (aortic and

carotid bodies) exert a marked reflex excitatory action on the medullary centers (respiratory and cardio-vascular centers) but do not seem to display similar effects on the cortex.

With respect to anoxia, it can be stated (122–124, 117, 118, 236, 30, 31, 198, 196, 92, 93, 17, 241, 75, 199, 91, 19, 109 *et al.*) that hypoxemia stimulates reflexly the respiratory and cardio-vascular centers by way of the aortic and carotid sinus chemoreceptors. These reflex impulses increase in frequency with increasing degree of hypoxia, as action potential studies of the chemoreceptor nerves have also demonstrated (236, 31, 91).

The great sensitivity of this reflex mechanism is attested, furthermore, by the fact that such chemoreceptor impulses could be recorded when the oxygen saturation of the blood fell to less than 96 per cent. Increase of oxygen saturation of the blood above 96 per cent decreases such chemoreceptor impulses.

Elimination of the chemoreceptors suppresses hyperventilation due to acute hypoxemia; the respiratory center, on the contrary, is directly depressed by acute oxygen want. Also the vasomotor medullary center is reflexly stimulated by hypoxemia through the chemoreceptors, while hypoxemia depresses the vasomotor directly. This is shown by the fact that the complete elimination of the chemoreceptors leads to a fall in blood pressure instead of the normal rise in blood pressure during acute anoxia (22, 83).

Whereas in acute anoxia, respiratory hyperventilation and increase in blood pressure depend solely on the reflex chemoreceptor drive, the increased respiration in chronic hypoxemia seems to be independent of chemoreceptor impulses and due to an increased activity of the respiratory center itself (19).

The suggestion has been made that impulses from the carotid and aortic bodies may also reflexly maintain cortical activity in the presence of anoxia (197). Syncope by anoxia might therefore result in those individuals who show little evidence of chemoreceptor stimulation in response to anoxia (55). Evidence bearing on this has been obtained in dogs by Randall (187).

The chemoreceptors of the sino-aortic regions are located in the carotid and aortic bodies. Contrary to the claims of Pi-Suner (182), no chemoreceptors sensitive to oxygen lack were found in the lungs.

Mott (170, 171) made histologic studies on brains of monkeys in which cerebral anemia had been produced. He found that after permanent ligation of the 4 main cerebral vessels, changes in cortical cells occur within 10 minutes which consist of slight swelling and chromatolysis. In animals that die within 24 hours, the cortical ganglion cells show diffuse staining, absence of Nissl bodies and coagulation necrosis. When signs of recovery are evident at the end of 24 hours, the cortical cells of such animals are swollen, the nucleus enlarged and displaced, but with some differentiation in the chromatic and achromatic substance remaining. Hill and Mott (130) repeated these experiments in cats and reported that in cats that die within 24 hours after brain anemia there is loss of chromatin granules and diffuse staining in cortical cells. Neurofibrilles showed no swelling. These workers concluded that large psychomotor cells of the cortex were more resistant to anemia than small pyramidal cells.

Crile and Dolley (49) made histological examinations on brains of dogs after presumptive recoveries and fatal cases induced by anemia. The neurocytes of the fatal cases uniformly presented the greatest change, not merely chromolytic but here and there definitely indicative of cell death. Marchi's method further supported the findings by proving the existence of fiber degeneration. The animals with best results in recovery ($7\frac{1}{2}$ minutes in time) apparently entirely returned to a normal state, had a degeneration of a number of fibers localized in the pyramidal fasciculi, traced from the cord to the cortex, and in Fleischig's fasciculus, as well as a more sparsely scattered degeneration of both ascending and descending fibers elsewhere. Gomez and Pike (96) killed cats at various periods after the occlusion of the cerebral vessels and reported a number of histologic changes in the brain. After periods of mild anoxemia, Martin, Loevehart and Bunting (168) found no histologic changes in the brain.

Spielmeyer (207) investigated the pathological changes in the brains of patients dying from various diseases which are supposed to produce cerebral anemia. He described lesions consisting of many shrunken, homogeneous, dark-staining cells that he considered characteristic of cerebral ischemia.

Gildea and Cobb (94) reported various typical histological changes in the brain of cats after sufficiently complete and prolonged cerebral anemia. Their experiments showed that periods of anemia of not more than 10 minutes result in permanent injury to the cortex, and not uncommonly in changes which end in death from convulsions and failure of the respiratory center. Therefore, 10 minutes of cerebral anemia is sufficient to impair cortical cells permanently. This figure may be high, because it is not probable that a complete cerebral anemia was produced in any of the animals used in these experiments.

Weinberger *et al.* (239), working with cats, reported permanent and severe pathological changes in the cerebral cortex at the end of 3 minutes and 10 seconds of cerebral anemia. Longer periods of anemic anoxia produced lesions in the Purkinje cells of the cerebellum and nerve cells of the basal ganglia.

Thorner and Lewy (224) reported that exposures to sublethal periods of pure anoxia produced vascular and degenerative changes in the central nervous system of guinea pigs. It was pointed out that these changes were irreversible. Lowenberg, Waggoner and Zbinden (161) reported lesions of the brain, especially in the cortex and basal ganglia, following fatal cases of nitrous oxide-oxygen anesthesia. These changes were attributed to anoxic anoxia. Van der Molen (227) suggested that cortical cell changes occur at partial pressures of oxygen equivalent to an altitude of 28,000 feet and, moreover, that some of these changes might be irreversible.

FUNCTIONS OF SPINAL CORD IN ARREST OF CIRCULATION

Stenon (210) reported that anemia of the spinal cord induces paralysis after one minute and suppression of sensitivity and motor functions after 3 minutes. Legallois (156) observed that ligature of the abdominal aorta induces paralysis of motor spinal functions, but that the spinal centers may recover their activity if the circulation has been reestablished after a given length of interruption. Brown-Séquard (33–36) reported that the spinal cord may recover normal functions after the

circulation has been restored after 40 minutes of interruption. Stenon (210) and Litten (159) showed that, in rabbits, prolonged anemia of the spinal cord induces deep and persistent lesions. Vulpian (237) and Schiffer (195) induced paraplegia of spinal origin after ligature of the abdominal aorta. Barbé (8) reported, for the most part, the persistence of spinal reflexes 3 to 8 minutes after decapitation. Spronck (208, 209) stated that the spinal centers are definitely paralyzed in rabbits 10 minutes after interruption of the blood supply. Colson (45) reported revival of spinal cord activities after interruption of circulation up to 20 minutes in dogs.

Experiments of King, Garrey and Bryan (142) showed that anoxia inhibits the knee jerk reflex. Porter (184) had shown earlier that anoxia may lead to the complete disappearance of the flexor reflex in spinal cats. Farber (63), Heymans, Bouckaert, Jourdan, Nowak and Farber (116) showed in dogs that the spinal vasomotor centers may restore their activity after arrest of circulation up to 25 minutes. Sauzède (193) observed that the spinal centers may revive in rabbits if circulation has been restored after 26 minutes of complete anemia.

Van Harreveld and Marmont (232) made observations of the functional recovery of the spinal cord from periods of complete anoxia. They asphyxiated cords of spinal cats by raising the dural pressure above the arterial. After long asphyxia (55 minutes) the tendon reflexes returned for 48 hours and then disappeared. Histologically, it could be shown that the anterior horncells were the most severely damaged structures of the spinal cord.

Gellhorn, Cortell and Carlson (77) showed that medullary excitation indicates an increased excitability of spinal centers in anoxia before these centers are paralyzed. Van Harreveld (228, 229) concluded that the average duration of asphyxia necessary to abolish all reflex activity in spinal cats is 3 minutes, 22 seconds; as a delayed effect of partial asphyxia this figure is increased to 11 minutes.

According to Häggqvist (105) constriction of the abdominal aorta for 15 to 25 minutes induces in the rabbit a permanent spastic paresis-inflexion of the hind limbs and degeneration of larger motoneurons, while the smaller anterior horncells and their axons in the root are largely spared. Van Harreveld (230, 231) reported that at the beginning of asphyxia of the cord, reflex activity is increased. The flexion reflex persists longer than the knee jerk. The knee jerk may be inhibited up to the time of its disappearance. Cooling the cord greatly reduces the rate of injury by asphyxia. Groat and Peele (102) recorded a large rise in arterial pressure when the cord was asphyxiated in decapitate cats. Experiments of Alexander (3) suggest that anoxia of the cord could account for this pressor response.

The metabolism of spinal cord tissue has been measured by Van Harreveld and Tyler (233) at intervals after asphyxiation of the cord for an hour. The oxygen uptake remains nearly normal for 6 hours and then drops over a period of 2 days to a level about 45 per cent of normal. These metabolic changes parallel the temporary return of reflex functions in these animals (232).

The histological changes occurring in the spinal cord after anemia have been investigated by several authors. Artificial anemia of the spinal cord was mostly induced by Stenon's modified method, i.e. passing a curved needle and thread around the spinal column at about the fourth lumbar vertebra, thus compressing the aorta against it, or by ligature of the abdominal aorta.

Spronck (209) believed that ligature of the aorta for half an hour is sufficient to produce necrosis and destruction of the cells of the spinal cord in rabbits. Ehrlich and Brieger (60) reported that one hour of ligation of the abdominal aorta produces death of the cord cells. The character of the histological lesions was much disputed (165, 136, 7, 192). Righetti (190) was the first to call attention to the differences in the lesions occurring after a temporary or a permanent anemia; in the latter the lesions appeared later.

The great resistance of the spinal ganglion to anemia was pointed out by Ehrlich and Brieger (60) and confirmed by de Buck and de Moor (52). After a temporary anemia of the cord, these investigators found around the nerve cells an infiltration of lymphoid cells which they believed to be phagocytes exuded from the lymph stream.

FUNCTIONS OF SYNAPSES AFTER ARREST OF CIRCULATION

Langendorff (150, 151), Langley (152, 153) and Hering (107) mentioned incidentally the exceptional resistance to anemia of cells belonging to the autonomic nervous system. Tuckett (225) induced anemia of the superior cervical ganglion and found that the ganglionic cells possess greater resistance than those of the spinal cord. The histologic changes within five days nearly correspond to the lesions in the cord after a maximum of 24 to 48 hours of anemia.

Schröder (200) seems to be the first to have studied systematically the effects of interruption of circulation in a sympathetic ganglion. He observed the long-lasting persistence of ganglionic activity after the death of the animal by bleeding and the possibility of restoring activity, once it had disappeared, by perfusion of the ganglion with Ringer's solution. Gomez and Pike (96), working with cats, reported histological changes in different nerve cells brought about by total anemia of the nervous system. They found that the order of susceptibility of the nerve cells to oxygen want, as shown by histological studies, was as follows: small pyramidal cells, Purkinje cells, cells of the medulla oblongata, cells of retina, cells of cervical cord, cells of lumbar cord, and sympathetic ganglion cells.

Cannon and Burket (39) pointed out the high resistance of the cells of the mesenteric plexus to complete anemia. Eccles (58) in a study of the electrical response of the superior ganglion, observed a decrease in the size of the electrical complex and a lengthening of the latent period following interruption of the circulation and remarked that a very minute blood supply is sufficient to maintain functional ganglionic activity.

Working on perfused ganglia, Bronk and Larrabee (28) observed an increase of the electrical response and a persistent facilitation in a ganglion deprived of its blood supply. They also reported, in working with the stellate ganglion, that it could be deprived of its circulation for about 30 minutes before it began to lose its capacity to respond; deprivation of its circulation for about 60 minutes, however, caused it to be functionless. Bargeton (10) investigated the effects of acute anemia on the superior cervical ganglion in cats during stimulation of the preganglionic fibers, with the nictitating membrane used as an indicator. He observed that the sympathetic ganglion cells show a striking resistance to deprivation of blood. A very slight blood supply is sufficient to keep the ganglion in normal activity. After the start of total

anemia, disappearance of activity takes from 10 to 15 minutes to become complete; it begins to reappear as soon as the circulation is restored, but the increase of functional response progresses more rapidly than the gradual decrease. Complete deprivation of blood as long as 70 minutes may still be followed by a large degree of functional recovery.

Sugar and Gerard (219, 220) pointed out that the synapse can withstand considerable anoxia. Bronk (25, 26) observed that after suppression of the blood supply for 6 hours, trans-synaptic conduction can be restored to a considerable extent by reestablishing the circulation. Even after such prolonged periods of anemia about 20 per cent of the fibers seem to conduct impulses across the synapse. It seems probable that sympathetic ganglia are characterized by a relatively low metabolism.

Bronk, Larrabee, Gaylor and Brink (27, 29) further investigated the effects of oxygen lack on the conduction of impulses along axons or on the transmission of impulses over synaptic pathways. When the oxygen supply to a ganglion is stopped by arresting the circulation, the transmission and excitation of impulses fail along more and more synaptic pathways until there is a complete failure of transmission through the ganglion within 30 to 40 minutes. The failure of transmission is due to oxygen lack; it is not the result of an accumulation of metabolites normally removed by the circulation. This is concluded from the observation that perfusion of the ganglion with oxygen-free solution causes failure of transmission as quickly as does arrest of perfusion or of natural circulation. An oxygen electrode brought into the ganglion shows that the oxygen tension within the ganglion falls to less than 5 per cent of its initial value within two minutes after stopping the circulation. Transmission over all ganglionic pathways continues for some minutes more, and over some pathways for 30 minutes. It follows that transmission is maintained for a considerable time in synapses with a very small rate of oxygen consumption or with no consumption whatever. Following the failure of conduction and trans-synaptic excitation in all of the neurons running through a ganglion, there is complete recovery if the circulation be restored, even though the anoxia has continued for as long as one hour. As many as 25 per cent of the synaptic pathways have recovered the ability to transmit impulses when the flow of fluid was restarted after $7\frac{1}{2}$ hours of interruption.

Using the method of the isolated perfused head of dogs, Heymans (111) reported that stimulation of the pre-ganglionic fibers of the superior cervical ganglion induces mydriasis during complete arrest of the circulation of the head up to 45 minutes. When perfusion is reeestablished, the mydriasis induced by pre-ganglionic stimulation reappears very soon, even after a complete interruption of perfusion up to 120 minutes' duration. Sympathetic axons, synapses, post-synaptic innervation and pupil thus are very resistant to anemia.

Gellhorn and Safford (85) determined the reactivity of the sympathetic-adrenal system to anoxia by determining the blood sugar changes produced by subjecting the animal to a pressure of 280 mm. Hg for 25 minutes. Repeated anoxia enhanced the response. Bonnet and Bremer (20, 21) led off synaptic potentials from the ventral and dorsal side of the frog's spinal cord during reflex action. The synaptic potentials are depressed by asphyxiation. Brooks and Eccles (32) performed similar investigations.

FUNCTIONS OF PERIPHERAL NERVES AFTER ARREST OF CIRCULATION

In 1859, Dubois-Reymond (206) reported survival of excitability of frog gastrocnemius for 10 days, when the muscle was kept at about 0° C. Brunquist (38) found that frog sartorius muscle immersed in Ringer's solution remains excitable for 8 to 142 days (average, 26 days) when kept at 5° to 6° C. Observations have been collected on the effects of oxygen lack on peripheral excitability and conductivity of frog nerves (68, 88, 89, 108). The average of survival times of nerves of *Rana pipiens* and *Rana catesbiana* are, respectively, 170 minutes and 230 minutes (243). The average survival time of Panulirus nerves is 76 minutes. The nerve activity of crayfish is unchanged after 5 and even 9 hours of anoxia. The lobster nerves in nitrogen are completely unresponsive to stimuli after $3\frac{1}{2}$ hours.

Sollmann (206) reported that the decline of nerve excitability of anoxic brachial and sciatic nerves of leopard frogs begins at median 7 days, extreme 15 days. The excitability of motor nerve fibers and endings survives almost as long as that of the muscle.

The survival times of mammalian nerves in anoxia were also determined. Lehmann (157) gives 25 to 35 minutes as the survival time for cat nerves, and Gerard (88, 89) reports 30 minutes survival time for a dog peroneal nerve. All the nerves taken from one rabbit or cat survived anoxia about equally long. Thus the nerve survival time is a characteristic of each individual animal. Though Lehmann (157) claimed that the saphenous nerve has a shorter survival time than other nerves examined, no evidence was found that correlation exists between specific nerves and survival times. According to Wright (243) the average survival time of 28 rabbit nerves was 24 minutes, of 10 cat nerves, 33 minutes and 3 dog nerves, 33 minutes.

Fabritius and Bermann (62) investigated the loss of different sensory modalities in a finger occluded at its base by an elastic band. It was shown that the stereognostic ability disappeared at a moment when touch and pressure still were felt. No sensations from touch and pressure was observed at a time when temperature and pain sensitivity still were intact. The ability of discriminating small differences in weight disappeared simultaneously with sensation for touch and pressure. Anesthesia appeared after an interruption of circulation of about 45 minutes.

Lewis, Pickering and Rothschild (158) carried out a systematic investigation on ischemic blocking of human nerves. One or several pneumatic blood pressure cuffs were applied to different parts of the arm and forearm for varying time periods and the sensitivity of the skin distal to the cuffs was tested. It was found that the anesthesia always starts in the distal part and spreads proximally. It was shown further that the different sensory modalities were blocked at different times, that the nerve is more sensitive to asphyxia in its proximal than in its distal part, and that long nerves are affected earlier than short nerves. These conclusions have been confirmed by most of the investigators (245, 222, 144, 101, 11) but questioned by some (16, 238, 203).

Thompson and Kimball (222) found that ischemia of the nerve first increased and later decreased the irritability of the fibers first stimulated by a current increasing from zero. They concluded that in mammalian nerves *in vivo* asphyxia affects the large fibers first. The authors, however, investigated low threshold fibers only, (i.e. large fibers) and did not test small fibers.

Zotterman (245) described ischemia experiments on the cat, and concluded that a nerve is more sensitive to asphyxia in its proximal part. After 40 minutes of ischemia many of the receptors and fibers were still in functional condition. Bentley and Schlapp (16) found that in the limb of a cat rendered ischemic the nerve in the leg and foot becomes inactive in about 30 minutes, though the nerve in the thigh survives for at least 2 hours.

Clarck, Hughes, and Gasser (44) investigated the electroneurogram during ischemia conditions. Later Gasser (73) investigated the blocking of different fiber groups in more detail. He stated that when a nerve is asphyxiated in an animal, the first fibers to be blocked are those in the delta elevation; then as the asphyxia progresses, the larger medullated fibers are included. In general, the larger the fiber the longer it survives, but the size-order is not rigidly followed. Adrian (2) reported that action potentials in the afferent fibers of the vagus nerve could still be registered up to one hour after cardiac arrest. Bayless (14) showed that the receptors and the fibers of the pulmonary vagus nerves involved in the respiratory Breuer-Hering reflex are still conducting up to 30 minutes of total arrest of circulation.

Frankenhaeuser (71) carried out a systematic investigation on the ischemic paralysis of a sensory nerve, the sural nerve, in the rabbit. The survival time was determined by recording sensory impulses by stimulating skin receptors. The survival time was found to be longer in short stretches of ischemic nerve than in long stretches. This difference was shown to be due to oxygen leakage. If leakage was prevented, long and short stretches survived for equal times. Thus no gradient in sensitivity to ischemia in the sural nerve was found. The proximal part of the sural area responded, however, some 3 to 7 minutes longer than the distal part. Fibers from slowly adapting receptors were blocked later than fibers from hair touch receptors, although the conduction rates of 'hair touch fibers' were shown to overlap those of 'slowly adapting fibers.'

Anoxia causes progressive depolarization and blockage of impulses in nerve tissues when depolarization reaches a critical degree (5, 244). An analysis of the process of anoxia of the nerve fiber of the frog revealed that for A fibers the average time to suppress activity is significantly smaller when both the stimulated and conducting regions are anoxiated, than when only the leading off section of the nerve is deprived of oxygen, as if the launching of the impulse were more easily suppressed than the blocking of conduction (38a). Worobjew (242), Tanaka and Toyosima (221) and Chauchard (41, 42) reported influences of anoxia and low atmospheric pressure on nerve chronaxia.

The effect of altitude on nervous excitability was investigated by Zwahlen and Granjean (246), Grandjean, Posternak and Waridel (98) and by Grandjean (97). In general the threshold of sensory structures was decreased, but that of motor nerves was unchanged. These changes were abolished by breathing O_2 at altitude, and could be produced by hypoxia at low altitude.

GENERAL THERAPEUTIC APPLICATIONS

Sudden circulatory arrest may occur in patients and may be due either to cardiac standstill or to ventricular fibrillation. The most essential therapeutic measure

in sudden circulatory arrest is the immediate restoration of the circulation. In some cases of sudden cardiac arrest, effective cardiac action may be promptly restored by cardiac massage (174, 235, 100, 164, 57). In ventricular fibrillation this is less likely to occur, and the circulation must be maintained by prompt manual propulsion of the blood by heart massage until defibrillation has been accomplished and forceful coordinated ventricular contractions occur again. At present there are two practical methods of interrupting ventricular fibrillation, namely the use of electric shock (15, 64) and administration of procane hydrochloride into the blood stream. The two methods may be used separately or as adjuncts to each other. Complete recovery after acute circulatory arrest has been reported using heart massage, electric shock and procane injections (191, 1, 174, 235, 100, 164, 57, 64, 149).

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ROLE OF METAL IONS IN ENZYME SYSTEMS[1]

A. L. LEHNINGER

From the Departments of Biochemistry and Surgery, University of Chicago
CHICAGO, ILLINOIS

MODERN BIOCHEMISTRY has made great strides in the identification of the components of enzyme systems and in the purification of enzyme proteins; few advances have been made, however, in understanding the nature of catalysis by enzyme proteins in chemical and physical terms. Those enzymes depending for their activity on metallic functional groups appear to offer an approach to these more fundamental problems since in many cases the catalytic activity of metalloenzymes is already present in a primitive form in simple inorganic compounds of the metallic moiety. The role of the protein moiety in enhancing these inherent properties of the metal is not yet known although recent advances in the field of the heme enzymes, for instance, offer considerable encouragement for the view that the role of the protein may eventually become comprehensible in terms of modern theories of molecular structure. In this article the role of metal ions in the action of various types of metalloenzymes is reviewed in the light of some general considerations on the physical properties of metallic ions as they relate to metal ion specificities among different metalloenzymes, and the nature of the structural linkages between metal ions and pertinent organic and inorganic molecules. It is not possible in the space allotted to cover all phases of this general subject nor can a complete compilation of the literature be made. Where possible, reference is made to a number of reviews and monographs which provide more complete documentation.

As an introduction it is appropriate to consider first the general role of metal ions in cellular physiology and the scope of their activities in terms of enzyme action. It is now a biochemical commonplace that the role of micronutrients and trace substances in cell physiology will ultimately be explained on the basis of their serving as active groups of various important enzymes (cf. 68). This view is, of course, well supported by the findings of the last 15 years of enzyme research, which have demonstrated, at least on a gross molecular basis, the role of several organic micronutrients such as thiamine, riboflavin, nicotinamide, pyridoxine and others in the action of certain enzyme systems. Equal progress has been made in identifying various trace metals as being required for the activity of a number of enzymes. In addition to the 'bulk' metals (K, Na, Ca, Mg, Fe) and the trace metals (Cu, Co, Mn, Zn, Mo) known to be essential in animal and plant nutrition, certain other metals such as Ba, V, Ni, As, Al, and Rb are present in trace amounts in various animal and plant tissues although it is not known whether or not they are nutritionally essential. Many of the enumerated bulk and trace metals have been found to be required in the action of one or another enzyme system or at the least have shown activating effects. However, the

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physiological significance of many of these observations may be questioned. For instance, arginase may be activated by Mn^{++}, Fe^{++}, Co^{++}, and Ni^{++} but by no other metals. Mn^{++}, however, is regarded as the physiological activating ion and the extent of physiological activation by the other ions is unknown. In any event observation of an activating effect by a metal *in vitro* does not necessarily define a physiological function of the metal in question. Although a number of studies have been made on the impairment of metalloenzyme activity in the tissues of metal-deficient animals (25, 93, 94, 197, 212), none of these have yielded completely decisive information on the nature of the primary enzymatic lesion immediately responsible for the metal deficiency syndrome.

It appears possible that physiological control over the activity of metalloenzymes may be effected by factors of metal ion availability and trace metal antagonisms. For instance, Roberts has shown that arginase exists in widely different states of metal activation in different animal tissues (178). Physiological trace metal antagonisms are not well known in terms of enzyme action, but it appears possible that antagonism on an enzyme level underlies effects such as that observed by Smith and Larson (211), namely, that the anemia produced by feeding zinc salts may be alleviated by administration of copper salts. In addition to these general comments on physiological control, it should be pointed out that the nutritional availability of a metal may condition certain ancillary but not essential cellular functions, as is illustrated by Pappenheimer's analysis of the role of iron in the nutrition of the diphtheria organism and in the production of the diphtheria toxin (169).

There is another aspect of the physiology of metallic ions which must ultimately gain the closest attention of the enzyme chemist. Among the most striking physiological phenomena are those of metal ion antagonism and selective distribution among the ions of the alkali and alkaline earth metals. Ringer's classical demonstration of the antagonism between K^+ and Ca^{++} and the requirement of Na^+ in sustaining the beat of the perfused heart and the numerous cases of selective distribution of Na^+ and K^+ serve as illustrations (cf. 85, 91). Recently, MacLeod and Snell have shown that the growth of certain microorganisms is dependent on alkali metal ions, and they have demonstrated a number of antagonistic effects among the metals of this series (137). Although none of the many ion antagonisms and selectivities of this type are as yet definitely ascribable to effects of these ions in known enzyme systems (or conversely, to effects of enzymes on these ions), it appears quite probable, judging from recent work on the chemistry of contractile systems (154, 222, 223), that these phenomena properly belong within the scope of metal-enzyme-substrate interactions. Of special interest to the chemist and physicist is the fact that not only the enzymatic (ATP-ase) activity of the contractile proteins of muscle but also the physical properties, such as flow birefringence and viscosity (i.e. the shape of the molecule), are controlled by antagonism between ions of the alkali and alkaline earth metals. The phenomenon of the selective distribution of Na^+ and K^+ in extra- and intracellular fluids may ultimately require the closest scrutiny of the chemist and physicist as well as the enzyme chemist for its explanation.

The role of metals in enzyme systems may be described with this broader viewpoint as involving 3 individual functions which are not necessarily mutually exclusive

(*1*). The metal may serve as the actual catalytic center of the enzyme, as it appears to do in the case of the heme enzymes, *2*) the metal may not be primarily involved in the catalysis, but may be required, as a binding group, to bring enzyme and substrate together in fruitful juxtaposition, and *3*) the metal ion may be required to maintain physiological control by antagonizing the activating effect of some other metal on an enzyme system. The contractile system of skeletal muscle is taken as a prototype in this case. The mechanisms of metal ion interaction in other processes, such as active motion of metal ions against concentration gradients and conduction phenomena, are still a matter of speculation but conceivably may involve functions in addition to those listed. The chemical and physical properties of metallic ions pertinent to their role in enzyme activity may now be considered.

CATALYSIS BY METAL IONS; MODEL SYSTEMS

It has been an entirely logical development that the enzyme chemist should turn to model catalytic systems not involving proteins for orientation. This approach dates back to the experiments of Edmund Davy in 1820 on the catalytic oxidation of ethanol to acetic acid in the presence of platinum and oxygen (which was a 'model' of the acetic fermentation) (cf. 244), and it has proven to be a most fruitful avenue to the understanding of enzyme action. It would appear that the usefulness of the enzyme model is a function of how well the model is understood in chemical and physical terms. Although Warburg's early experiments on the catalysis of amino acid oxidation by blood charcoal appeared at the time in the minds of many to be far removed from the physiological problem of respiration, we can now only admire the subsequent developments in his and other laboratories which have since made the physiological action of the heme proteins reasonably comprehensible in terms of their structure. It is not possible to consider here the interesting history of model metal catalyses as they apply to biological oxidations; the monographs of Oppenheimer and Stern (168) Warburg (244) and Schwab (190) may be referred to in this connection.

The catalytic activities of some metalloenzymes are already inherently present in a primitive form in the metallic moiety. The different functions of heme proteins, such as electron transport, decomposition of hydrogen peroxide, and peroxidatic activity, are already evident in simple iron salts. One or another of these properties is selectively enhanced in the various heme enzymes by various structural adaptations which are discussed later. The enzymatic activities of the copper enzymes and the oxaloacetic and oxalosuccinic carboxylases are also inherently present in the metallic moiety. In these cases the role of the protein is not yet clear, but in the case of some of the heme proteins, at least, it has been found that the histidine groups of the protein, which coordinate with the iron, play a special role in the function of the heme which has been interpreted in terms of specific resonance hybrids of the imidazole group and for which Hearon *et al.* have provided a most instructive model (83, 84). In the study of model catalyses by metal ions it will, of course, be most important to make use of the modern concepts of the nature of catalysis and experimental approaches to catalytic phenomena afforded by the theories and investigations of Bronsted, Pedersen, Schwab and others.

It appears significant that no model catalyses are known for many metal-activated enzymes. For instance although carbonic anhydrase contains zinc, which is absolutely specific and necessary for activity, no other zinc compound shows even feeble activity in catalyzing the reaction (110). In the case of arginase, a Mn^{++}-requiring enzyme, Mn^{++} compounds have no catalytic activity in the absence of the enzyme protein (89). A number of peptidases require metal ions for activity, but no model metal ion catalysis has been described for the hydrolysis of the peptide bond, although it must be pointed out that in this and other cases perhaps no intensive search has been made for such model catalytic systems, which may involve somewhat different conditions than the enzymatic catalysis. It is tempting to speculate that the existence or non-existence of a model metal catalysis for a given enzyme may have meaning in terms of the mechanism of action of the metal. For instance in the case of a metalloenzyme for which a model metal-ion catalysis, however feeble, is known it may be argued that the metal is the catalytically active component of the enzyme and that the protein has the role of enhancing the inherent catalytic property of the metal ion by exerting binding or orientation forces on the substrate, etc. In the case of metal-requiring enzymes for which a thorough search does not reveal a model non-enzymatic catalysis involving the same or a related metal, it may be postulated that the metal is not the active catalytic center of the enzyme but may have the role of bringing the substrate molecule close to a catalytic site on the protein molecule by virtue of its ability to form, for instance, an asymmetrical coordination complex. In this case the protein would supply the catalytic center. There is as yet insufficient information available to pursue this idea further with profit.

PROPERTIES OF METALLIC IONS AND THEIR BIOLOGICAL SPECIFICITY

Apart from the relatively few metalloenzymes showing absolute metal specificity, such as the heme enzymes, copper enzymes, carbonic anhydrase, etc., there is a large group of enzymes in which the metal specificity is less sharp or even diffuse and in which the degree of dissociation of the metal-enzyme complex is usually relatively high. This group includes the exopeptidases, keto acid carboxylases, arginase, the different phosphatases and phosphate-transferring enzymes (including the actomyosin system). Among the enzymes of this group there are many cases in which more than one metal ion may activate, and there are also some examples of inhibition by metals, some of them competitive in nature. The metal ion antagonism shown by the actomyosin system, of course, is of especial interest. This latter group, then, presents the problem of accounting for the metal ion specificities and antagonisms in chemical and physical terms and if, by extension, we may include phenomena of selective distribution of ions, it will be seen that the question embraces not only the specificity of the metal in simple enzyme systems but a large area of physiological phenomena as well.

Competitive antagonism to various organic substrates of the cell such as vitamins, metabolites, hormones, etc. is now being extensively and successfully studied from the standpoint of the structural relationship between substrate and antagonist (179, 251) and these structural relationships, such as in the sulfonamide-p-aminoben-

zoic acid competition, have on the whole been clearly obvious. However, little attention has been given to applying such concepts of structural requirements to metal ion specificities and antagonisms, although the general problem of metal antagonisms and selectivities has occupied the attention of physiologists for many years: consider the work of Ringer, Loeb, Lillie, Höber, Osterhout, Heilbrunn and others. Although some theories to account for metal ion selectivities have been proposed (85, 91, 194), these have not usually made use of the full extent of modern knowledge of the structural and chemical properties of metallic ions in solution.

An approach to the problem of the physical basis of the specificity of metal ions in enzyme systems and the phenomena of selectivity and antagonism might be made by first considering some of the immediately obvious parameters of ion structure and properties (it will be seen that these are not all completely independent factors). These may be listed: *1*) mass, *2*) ionic charge, *3*) ionic radius (this may be approximated from crystallographic data (170), although it is certain that crystal ionic radii are probably not identical with radii of ions in solution), *4*) the potentiality of reversible valence change and the electrode potential of such a couple, *5*) rate of diffusion, *6*) mobility in an electrical field, *7*) the configuration and stability of the hydrates of the metallic ion in solution and *8*) the configuration and stability of coordination complexes of the metallic ion with substances other than water (i.e. substrates, proteins, etc.). Factors *7* and *8* are not entirely predictable from readily measured physical properties; however, the firmness of hydration and coordination is to a large extent dependent on the ratio of charge to ionic radius, the so-called ionic potential (50). Ultimately, of course, the characteristics of coordination complexes of metals with water or other polar molecules will be a reflection of the electron configuration of the metallic ion and the coordinated groups. It should also be pointed out that the energies and entropies of complex formation will be critical factors because they determine the ease of replacement of a coordinated group by some other molecule (cf. 81, 127). The above is not necessarily a complete list of pertinent factors but will suffice as a first approximation. Details concerning these properties or actual data are furnished by some general references (50, 56, 81, 127). It should be pointed out here, and this will be discussed at greater length later, that the role of factors *7* and *8* (7–9) cannot be determined with certainty at the present time because of some deficiencies in present knowledge.

It is instructive, however, to consider a few cases of enzyme specificity for metal ions in the light of the factors enumerated above. Arginase, for instance, may be activated *in vitro* by Mn^{++}, Fe^{++}, Co^{++}, and Ni^{++} and no other ions; Mn^{++} is regarded as the natural activator. The 4 activating ions are closely related since they are members of the first transition series of the periodic table. They therefore differ little in atomic weight, ionic radius and potential, mobility etc. and are equal in charge. They are all capable of forming coordination complexes with a large variety of organic functional groups, and these may be either ionic or of the covalent type. Since the degree of filling of the *3d* electron orbitals differs in these metals the coordination complexes they form may differ in electron configuration and type of linkage, and it is conceivable that the differences among these metals as to rates of arginase activation (151) may depend on the latter factor. On the whole this superficial analysis

on the basis of the enumerated factors appears reasonably satisfactory and it might be predicted that these particular transition metal ions might show a certain degree of similarity in their ability to serve as activating ions for enzymes other than arginase. Exceptions to this generalization are, however, immediately obvious. For instance, iron is the only metal found in any abundance in animal tissues in coordination with porphyrins, although many other metals may form very stable complexes with the porphyrins and at least two (Mn, Co) may form reversible oxidation-reduction systems (65). In the case of an enzyme with less sharp metal specificity, leucine aminopeptidase, Mn^{++} and Mg^{++} are the only metal ions showing activity, Mg^{++} being considerably less active than Mn^{++}. The difference between Mn^{++} and Mg^{++} with respect to mass, ionic radius, ionic potential, mobility, and rate of diffusion is generally considerably greater than between Mn^{++} and other transition metals (in divalent form). Factors *7* and *8*, which concern the ability to form coordination complexes with H_2O and other substances, cannot be evaluated with complete certainty although it is probable that both Mn^{++} and Mg^{++} form aquocations of the type $M(H_2O)_6^{++}$ (as do Fe^{++}, Ni^{++} and Co^{++}) and both may form coordination complexes with organic compounds, although Mn^{++} would appear to be more versatile in this respect since the unfilled *3d* electron orbitals allow formation of covalent as well as essentially ionic complexes. In the latter instance there is again more similarity between Mn^{++} and the other transition metal ions than between Mn^{++} and Mg^{++}. This superficial analysis does not appear to offer an immediately obvious explanation for the enzymatic specificity for Mn^{++} and Mg^{++} and the inactivity of other metals. This particular metal specificity is not an isolated case since a Mg^{++}-Mn^{++} similarity is evident in a number of phosphatases, phosphate-transferring enzymes and some of the keto acid carboxylases.

The ions of a given vertical group of the periodic table (such as the alkali metals or alkaline earths) might be expected to differ greatly in their ability to replace each other in activation of a metalloenzyme, since the variations of mass, ionic radius and ionic potential (which to some extent determines ability to form coordination complexes in these 2 groups of metals (50)) are large, especially at the extremes. Not only are such differences in specificity commonly seen, but also it actually appears as if antagonistic pairs of ions are best sought within a given vertical group. Na^+ and K^+ selectivities are well known; alkaline phosphatase, probably a Mg-protein, is strongly inhibited by Be^{++} (47, 77, 114). Other examples might be pointed out among the phosphatases and phosphate-transferring enzymes. Work of MacLeod and Snell already quoted (137) shows very striking antagonisms between alkali metal ions. If the Be^{++}-Mg^{++} antagonism is examined from the standpoint of the factors of ion structure and reactivity enumerated, it again does not appear immediately obvious as to why the lighter, smaller, much more firmly hydrated Be^{++} ion should be such a potent antagonist for Mg^{++} when the more nearly similar Ca^{++} is only weakly antagonistic, if at all, to the action of Mg^{++} in alkaline phosphatase.

Although the preceding discussion indicates a certain degree of dependence of ion specificity on factors of mass and dimensions, it is clear that it is probably not possible to provide a reasonable explanation for ion specificity and antagonism on the basis of the properties of the 'naked' metallic ion. The great unknown quantities are the

factors of hydration and complex formation. Since the metallic ion in solution in a physiological milieu is surrounded by an atmosphere of other molecules, such as loosely bound water molecules or more tightly bound organic and inorganic substances of polar nature, it is conceivable that the 'isostere' concept may be more properly applied to the structure of the entire metal ion complex, rather than the naked ion. This, again, is probably an oversimplification. In any event it becomes appropriate to summarize briefly present views on the structure of coordination complexes and some of the major problems as they pertain to the questions raised in this discussion.

INTERACTION OF METAL IONS WITH PROTEINS AND SUBSTRATE MOLECULES

Two types of bonds are involved in metal ion reactions with organic molecules, ionic and covalent. The ion-pair 'bond' resulting from electrostatic attraction between two oppositely charged ions is manifested normally only in crystal structure. This type of interionic attraction could conceivably be involved, however, in a structurally organized system as in the actomyosin complex, the physical order of which is dependent on the presence of certain ions. A second type of electrostatic bonding of more general importance is the ion-dipole bond (170) which results from electrostatic attraction between the positively charged metal ion and a dipolar molecule, whether permanent or induced. Many such ion-dipole complexes involving metal ions are known, ranging from the simple aquocations such as $Mg(H_2O)_6^{++}$, $Cu(H_2O)_4^{++}$, and possibly $Na(H_2O)_4^{++}$ to very complex forms such as the ionic iron complex hemoglobin. The ability of metallic ions to form such ionic complexes generally increases with the so-called ionic potential (50), with the exception of the ions of the transition series, which show ability to form complexes out of all proportion to their ionic potential and which, for other reasons, must be considered as a separate class below. Ions of low ionic potential such as Cs^+, Rb^+, and K^+ show least tendency to hydrate or form complexes, those of somewhat higher potential, such as Na^+ and Ba^{++} show intermediate activity, and those of high potential such as Be^{++} and Al^{+++} show extreme readiness to form stable complexes. The transition metals are far more active in forming coordination complexes; they differ in that they may form essentially covalent linkages between metal and the coordinated molecule as well as ionic complexes. The difference is due to the fact that in the metals of the first transition series (Ti, V, Cr, Mn, Fe, Co, Ni, Cu^{++}) the $3d$ electron orbitals are not completely filled due to relatively low differences in energy levels between the $3d$ and $4s$ orbitals (cf. 50). These metals have a tendency to borrow electrons from other molecules to fill out these orbitals, thereby establishing essentially covalent linkages in which a pair of electrons is shared between metal and the group bound. The transition metals may also form ionic complexes. It must be most strongly emphasized at this point that the distinction between ionic and covalent complexes drawn above represents a gross oversimplification of a very complex situation. Actually many coordination complexes have properties which suggest that the bond must be considered as an intermediate hybrid between the two extremes of bond types. For this reason some authorities prefer not to use the terms ionic and covalent but rather the older terminology of Biltz (50), which is based largely on the stability of the complex. Thus, very firmly bonded complexes are called 'penetration' complexes and the more loosely

bound are called 'non-penetration' or 'normal' complexes. Although the terms 'ionic' and 'covalent' will be used in this review the above reservations should be held in mind.

A special property of transition metal ions, of great experimental usefulness, is the magnetic behavior. This derives, in the first transition series, from the incompletely filled *3d* orbitals. The presence of unpaired electrons, which possess unopposed spin moments, in these orbitals confers the property of paramagnetism. A completely closed or filled configuration as is found in metal ions outside of the transition series is diamagnetic. The electron configuration in the *3d* orbitals also determines in large part the characteristic absorption spectra of these metals and their complexes.

The metal ion may coordinate with a number of molecules of a substance such as ammonia or H_2O and the coordination number indicates the number of groups coordinated. Definite spatial configurations result: planar and tetrahedral among 4-coordinated metals, and octahedral among 6-coordinated metals although 'forced' configurations are known. Coordination may take place with 2 susceptible groups of the same molecule to form a bidentate chelate complex, or less usually with molecules containing 3 or 4 groups appropriately situated to form tridentate and quadridentate chelate complexes. In the formation of chelate complexes only configurations leading to relatively strain-free rings take part, 5- or 6-membered rings being most stable. Polydentate complexes are generally more stable than unidentate complexes and some show extremely low dissociation. For instance, the iron of ferriheme and ferrihemoglobin (which are quadridentate, ionic complexes) was found not to exchange significantly with Fe^{+++} labeled with radioiron when they were present in aqueous solution together for a period of 2 months (185). General reference works should be consulted for further details on coordination complexes (50, 56).

Although many coordination complexes of metals with organic molecules have been prepared and studied in detail (50, 56), actually very little is known concerning the nature and stability of complexes of *physiologically* important metal ions with *physiologically* important substances such as proteins, amino acids, peptides, keto and hydroxy acids, phosphorylated compounds, etc. under *physiological* conditions of pH, concentration and temperature. The chemist has been largely concerned with relatively stable metal chelates which may be isolated in pure form. Although some chelates of high stability exist biologically (hemes, chlorophylls), by and large chelate complexes of biological interest may be presumed to be *relatively* highly dissociated under physiological conditions, especially those involving alkali and alkaline earth metal ions. The reactions of metal ions with protein molecules have been studied for many years beginning with the work of Hofmeister. Schmidt (187) and Greenberg (72) have reviewed much of this work. However, little has been done on the nature of the linkages between metal and protein and the functional groups involved. The recent study of Klotz and Curme (115), employing equilibrium dialysis, offers an approach which promises to yield considerable information on this type of complex. Complexes of metals with amino acids have been known for some time; the papers of Hearon (83, 84) refer to much of the literature on this subject. There is however very little information on complexes involving peptides and especially asymmetrical

polynuclear chelates of the type proposed by Smith (210) as being involved in peptidase action. Information is especially scanty on the alkali and alkaline earth metal complexes. Complexes of this type usually have high dissociation constants and are in readily reversible equilibrium with the free metallic ion. These complexes have presented the formidable technical difficulty of measurement of the free metallic ion in equilibrium with the complex without disturbing the equilibrium. For instance, for the measurement of free Ca^{++} in the presence of Ca^{++}-complexes McLean and Hastings (136) found it necessary to employ bioassay of free Ca^{++} by means of its effect on the perfused frog heart. Some data on the complexes of Ca^{++} and Mg^{++} with a few organic acids and amino acids have been collected by Davies, Cannan, Greenwald and their colleagues (32, 73, 74, 235). The crying need is for new analytical methods which will permit measurement of equilibria and stability of easily-dissociated metal chelates under different conditions. Recently Calvin and his associates have studied the stability of a series of metal chelates employing systematically such techniques as the use of isotopic metals, polarographic estimations, and acid-base titration methods (31). Schwarzenbach and his colleagues have also studied stability of chelates in solution (4, 191). Perhaps the most significant recent advance in the measurement of complex ion equilibria is the work of Schubert and colleagues (189a), who have devised a means of studying such equilibria which involves the use of essentially carrier-free isotopic metals and cation exchange resins, which bind only the free metallic ion and thus yield a measure of the free ion in equilibrium with ligand molecules. The extreme flexibility of the method, which allows measurements at widely varying pH values and ligand concentrations, promises that it may become the method of choice for the investigation of loosely bound complexes of biological interest.

It is not sufficiently appreciated by the biologist that considerable complex formation may even take place between metallic ions and anions such as $HPO_4^=$, $SO_4^=$, etc. Greenwald and Hastings and their colleagues have studied the apparent dissociations of $CaHPO_4$ and $MgHPO_4$ and have found them to be quite low (75, 226). It is evident that interionic attractions of one kind or another must account for these low values. Some experiments from the physical chemical literature strikingly illustrate the extent of complex ion formation among alkali and alkaline earth ions. McBain and Van Rysselberge (135) have found that when a solution containing $MgSO_4$ and K_2SO_4 is subjected to electrolysis the bulk of the Mg moves toward the anode, indicating that the Mg is in the form of an anion. Similarly, they have presented evidence that the electrophoretic movement of K^+ may be suppressed in the presence of Na^+. Conditions of these experiments were not far removed from physiological and they indicate a degree of interionic attractions not generally appreciated by the biologist. These very unstable, probably unisolatable complexes may ultimately prove to be of considerable biological significance. In any case it appears from the foregoing that considerable work remains to be done on the nature and properties of coordination complexes of metal ions as they bear on the general problem discussed here.

These general considerations on the properties of metal ions serve as an introduction to a discussion of the action of the reasonably well-known metalloenzymes.

It is hoped that this introduction will underline some major problems which remain to be approached in the study of metalloenzyme action.

HEME ENZYMES

Wyman has stated that the heme proteins "represent, perhaps at its acme, the instance of organic adaptation at a molecular level" (252). True as this statement is for the case of hemoglobin, it must, however, be pointed out that this impressive relationship between structure and function of hemoglobin is very largely a matter of understanding, based on an enormous and well-erected structure of facts. Perhaps, with the case of hemoglobin to furnish a standard, it may be possible in the future to define function in terms of even more exquisite structural adaptation not only for purely enzymatic functions of heme proteins but also for other metalloenzymes as well. A number of excellent reviews and monographs on the heme proteins and related subjects exist (5, 65, 82, 132, 150, 168, 184, 221, 231, 244, 252), which may be consulted for details of properties, structure and enzymatic activity. The reviews of Wyman (252) and Theorell (231) are especially comprehensive. The brief treatment which follows is concerned largely with the role of iron in catalysis, the state of the iron atom in the different heme enzymes, and the nature of the linkages to porphyrin, protein, and oxygen or other substrates. Some recent advances of significance are pointed out.

The different catalytic properties of the heme proteins are already inherently present in simple compounds of iron. For instance, iron salts have long been known to catalyze the decomposition of H_2O_2 in the absence of (catalase activity) and in the presence of (peroxidase activity) oxygen acceptors (sugars, fatty acids, amino acids, thiols, etc.). Iron salts also catalyze oxidation of thiols, ascorbic acid, phenols, etc. by molecular oxygen (oxidase action) and the ferricyanide ion may accept electrons to form ferrocyanide (electron transport function (65, 168, 217). The enhancement of these different inherent properties of iron as they appear in the heme enzymes is obviously a selective one and, as will be seen in the following discussion, the structural basis for the specificities of the heme enzymes is gradually revealing itself. Although heavy metals other than iron are known to catalyze one or more of these reactions and many metals may form firmly bonded complexes with porphyrins, only iron and magnesium porphyrins have been found in nature in any abundance (65).

Before discussing the individual heme proteins some fundamentals of heme and heme protein structure may be pointed out. The 4 pyrroles and the iron atom are probably coplanar, by analogy with the metal-phthalocyanines, which have been extensively studied by x-ray analysis by Robertson (cf. 252). The fifth and sixth coordination bonds are at right angles to the plane, one above and one below. The coordination of iron with the pyrrole nitrogens involves displacement of 2 protons; ferroheme has no net charge whereas ferriheme has a single positive charge. One of the two other coordination places may be open for combination with O_2, hydrogen peroxide, carbon monoxide, cyanide, etc., and the other is taken by certain groups of the protein; in cytochrome-*c* both the fifth and sixth positions are taken up by the protein. Hemoglobin and catalase are poly-heme proteins in which there

is some evidence of heme-heme interactions; cytochrome-*c* and some of the peroxidases are monoheme proteins.

The state of the iron atom and the nature of its linkages with the various components of purified heme proteins have been studied with 3 main approaches which have largely supplemented and confirmed each other: namely, acid-base and redox titrations, spectrophotometric examination and magnetometric investigations. While it is not the intention to minimize the importance of the other approaches and the contributions of many distinguished investigators, it is intended to stress the magnetometric approach, because it has been most revealing as to the function of the iron atom in terms of modern structural concepts. The application of this method to hemoglobin actually dates back many years (61, 252), but Pauling and Coryell (171) were the first to use it with reference to modern quantum theory. Full understanding of the significance of these measurements requires some background of knowledge of the configuration of electrons surrounding the iron atom and the properties of electrons in their different loci and energy states (82, 195). Briefly, substances containing no unpaired electrons or permanent magnetic moment are diamagnetic, whereas those containing unpaired electrons are paramagnetic due largely to the unopposed spin momentum of the unpaired electrons. These properties are measurable in terms of magnetic susceptibility. Pauling and Coryell demonstrated that the oxygenation of hemoglobin causes a change from the paramagnetic to the diamagnetic condition. This observation is more striking when it is considered that the oxygen molecule in its free state is itself paramagnetic due to the presence of 2 unpaired electrons. The observed magnetic susceptibility of hemoglobin, some 5.4 Bohr magnetons per heme, indicates that the complex has 4 unpaired electrons (Pauling has recently discussed some discrepancies (184)) and that the bonds between the iron and the 4 pertinent nitrogen atoms of the porphyrin and the coordinated group of the globin (the imidazole group of histidine) (252) are essentially ionic. In ferrihemoglobin the magnetic data indicate 5 unpaired electrons per heme and in this case also essentially ionic binding with porphyrin and globin. Oxyhemoglobin and carbon monoxide hemoglobin, however, were found to be diamagnetic. Since both the ferrous ion and the oxygen atom contain unpaired electrons, interaction of molecular oxygen with the hemoglobin must therefore have resulted in a complete electronic rearrangement, so that the unpaired electrons of oxygen and iron now pair; the spin moments of the electrons are opposed and cancel out, resulting in loss of paramagnetism. Similar changes occur in the formation of carbon monoxide hemoglobin. This change in the character of the bonds on oxygenation also underlies the Bohr effect, namely, the fact that oxyhemoglobin is a stronger acid than hemoglobin. Coryell and Pauling (40) have provided an interpretation for the Bohr effect based on a shift in the character of the bond between iron and the imidazole nitrogen caused by the electron rearrangement on oxygenation. Although space limitations prevent full discussion of their interpretations (these are discussed at length by Wyman (252)), it must be concluded that the Bohr effect, so important in the physiology of respiratory exchange, is a special function of the nature of the bond between iron and the imidazole group of histidine and, most important, is possible only because of the fact that the imidazole group may exist as two or more different types of resonance hybrids depending on the na-

ture of the coordination with iron, which in turn is determined by oxygenation. The participation of histidine in this molecular adaptation is specific; hemoglobin would probably not possess one of its most important properties if the linkage of iron to globin involved, for instance, an α-amino group instead of the imidazole group of histidine. The importance of the histidine linkage is also brought out in the study of cobaltodihistidine by Hearon which is discussed later. The sigmoid oxygen saturation curve of hemoglobin appears to be due to heme-heme interactions; the monoheme protein myoglobin shows the expected hyperbolic oxygen saturation curve (252).

Theorell (231) has pointed out that so far no adequate explanation exists for the fact that hemoglobin may take up oxygen reversibly without affecting the valence state of the ferrous ion and he suggests that the differences among heme proteins in this respect may be ascribed to the linkages between heme and protein. After oxygen has coordinated at the sixth coordination position of the iron, it does not appear possible to accept electrons from the iron, and this stabilization appears to be conditioned by the imidazole linkage and the resonance stability of the imidazole group. If the latter linkage is broken by denaturing the globin, oxidation of the ferrous iron by the coordinated oxygen takes place (231). This may be regarded as a model of cytochrome oxidase action.

Cytochrome-*c* differs from hemoglobin in that it is not autoxidizable and does not form compounds with carbon monoxide, fluoride and cyanide in the physiological pH range. Although it has been reported that cyanide does combine with ferricytochrome-*c* the reaction is very slow (cf. 231). Another point of difference is that the iron undergoes reversible valence change during its physiological function. In explanation of these properties the following facts have been adduced by Theorell (231): titration curves of cytochrome-*c* show that the dissociation constants of the imidazoles of the 3 histidine residues are partly displaced from their normal range, indicating that 2 imidazoles are probably coordinated with the iron in the physiological pH range. If 2 imidazoles are bound to iron, the latter has no more coordination places open for reaction with oxygen, carbon monoxide, cyanide, etc. At low pH values titration data show that one of the 2 heme-linked groups is dissociated. Significantly, it is only under these conditions of extreme pH that the cytochrome may react with oxygen, cyanide, carbon monoxide, etc. Magnetometric measurements show that ferrocytochrome-*c* is diamagnetic, therefore indicating that the iron is held by essentially covalent linkages to both porphyrin and imidazoles. Ferricytochrome-*c* at physiological pH values is paramagnetic and the magnetic susceptibility indicates only one unpaired electron, all other bonds being covalent. It may be concluded then that the only change during oxidation and reduction of cytochrome-*c* is the loss or gain of a single unpaired electron. Theorell has pictured the heme as being built into a crevice of the protein, the fifth and sixth coordination places being firmly bonded (covalent), probably to imidazole groups of the protein, accounting for the inertness toward O_2 and CO because of the inaccessibility of a coordination position. Theorell has suggested that the imidazole group may act as the electron 'transmitter' to the iron since it may accept an electron to become a free radical. Change in the resonance structures of the imidazole then may cause transfer of the electron to the iron atom.

It should be pointed out that the porphyrin moiety of cytochrome-*c* may itself also conceivably act as an electron transmitter since it exists as a conjugated system.

The peroxidases (5, 35, 231) which catalyze the reaction between hydrogen peroxide and a number of oxidizable acceptors such as ascorbic acid, leuco malachite green, iodide, etc., act in the ferric state without reversible valence change. Carbon monoxide does not inhibit peroxidase. However, peroxidase may act as an oxidase (i.e. reversible ferrous-ferric valence change, ability to react with oxygen), when dihydroxymaleic acid is the substrate. This reaction is inhibited by carbon monoxide, indicating a reversible valence change of iron. The acceptor specificity of the peroxidases is quite broad and includes, at least in the case of horse-radish peroxidase, ferrocytochrome-*c*. Hogness and his colleagues (9) have described a cytochrome-*c* peroxidase for which ferrocytochrome-*c* is the specific acceptor. Recently, Chance (35) has found that the horse-radish peroxidase-hydrogen peroxide complex, contrary to Hogness and his colleagues, may also react very readily with ferrocytochrome-*c*, suggesting that all peroxidases may have this property. The significance of this reaction in biological electron transport is not clear. Magnetometric measurements of ferriperoxidase by Theorell (231) indicate 5 unpaired electrons in the physiological pH zone. This means that the ferric ion is ionically bound to porphyrin and to the protein moiety; this configuration resembles that of ferrihemoglobin. Peroxidase reacts readily with cyanide, nitric oxide, azide, sulfide and fluoride, (as well as with hydrogen peroxide) indicating at least one position open for coordination with substrate or competing substance. It appears improbable, from titration data, that imidazole groups are involved in the bond with protein. Theorell has suggested that a carboxyl group in ionic linkage is the mode of attachment.

Peroxidase forms at least 3 different spectrophotometrically observable complexes with hydrogen peroxide (cf. 35, 231). Chance (35) has shown that complex I, an essentially ionic complex, spontaneously changes into the essentially covalent complex II (they differ in absorption spectra and stability). Complex II is the form which reacts with the acceptor.

Although catalase has been intensively studied it has been difficult to obtain definitive data on the mode of combination of iron with the porphyrin, protein and substrate. Theorell has outlined these difficulties (231) and it appears that the success of Bonnichsen (23) in obtaining catalase free of extraneous biliverdin will allow more significant spectrophotometric measurements. It is quite probable (231) that the iron is bound to porphyrin and protein by essentially ionic linkages. The linkage with the protein is not known in detail. The sixth coordination position is occupied by a hydroxyl group, which may be displaced by hydrogen peroxide or by a variety of anions which are known to be inhibitory. It is this position which reacts with hydrogen peroxide to give the intermediate complex. Although Keilin and Hartree had proposed a mechanism of action involving a cyclic ferric-ferrous change (106) the best evidence now indicates that the iron stays in the ferric form during catalase action (217, 221, 231). The mode of catalysis of peroxide decomposition by iron compounds has been intensively studied although a definitive mechanism has not yet been agreed upon due to the rather complex kinetics of hydrogen peroxide decomposition. Recently George, in Keilin's laboratory, has studied the kinetics of this catalysis by

various iron compounds (62, 63). Keilin and Hartree have shown (107) that catalase may activate an oxidation of alcohols by H_2O_2; their work and that of Chance (33) indicates that this may be regarded as a peroxidatic function of catalase, since the complexes of peroxidase and catalase with hydrogen peroxide show many similarities. The differences in acceptor specificity between true peroxidases and the peroxidatic function of catalase may be due to a relative inaccessibility of catalase hematin to large molecules while the peroxidase hematin is accessible to as large a molecule as cytochrome-*c* (35).

The investigations of Chance (33–35) on the spectrophotometric observation of intermediate complexes of catalase and peroxidase of very short half-life by means of refinements of the flow technique represent a great advance in enzyme chemistry. Not only has this method yielded direct evidence for the existence and structure of several enzyme-substrate complexes but also it has allowed determination of the velocity constants of these different complexes in their different reactions. Another point of interest approachable by this method is the question of the existence and nature of interactions between the different hemes of the catalase molecule. For instance, Chance has found that although the primary complex of hydrogen peroxide and catalase involves only one heme, the other hemes must be free for maximum catalytic activity (34).

Little can be said in detail concerning the nature of the 'atmungs-ferment,' or cytochrome oxidase. The classical investigations by Warburg (244) on the action spectrum of the photodissociation of the inactive carbon monoxide complex, studied in intact cells, have revealed that a heme enzyme is involved, in which the heme is of the red-green variety. Warburg has calculated the molecular weight of the apoprotein of the respiratory ferment to be approximately 75,000 per heme (243) by making use of the recent finding of Bücher and Kaspers (28) in his laboratory that the quantum yield in the photodissociation of carbon monoxide myoglobin is constant and independent of wave length (vide infra).

It is obvious that the respiratory ferment must undergo cyclic $Fe^{+++} \rightleftharpoons Fe^{++}$ changes since it accepts electrons from the cytochromes and the Fe^{++} form must react with oxygen to regenerate the Fe^{+++} form. These two properties are not seen together in any other of the well-known heme proteins. Presumably the ferrous form may be oxygenated, as in oxyhemoglobin, since carbon monoxide (which combines only with ferrous iron) is a competitive inhibitor. The oxygenated ferro enzyme probably undergoes an intramolecular change in which an electron moves from iron to oxygen. This does not occur in oxyhemoglobin and it will be recalled that this remarkable stability of oxyhemoglobin may be conditioned by the nature of the heme-protein linkage. It may be expected, then, that the heme-protein linkage in the respiratory enzyme may be the key to its special properties. Theorell and Swedin (230) have shown that peroxidase may show some of the qualities of an aerobic oxidase when it acts on dihydroxymaleic acid as substrate. The oxidation is inhibited by carbon monoxide and the inhibition is light-sensitive, properties not shown by the true peroxidase reaction. Hydrogen peroxide is, however, necessary in the reaction, which is inhibited by catalase, and therefore this action of peroxidase is probably not a true oxidase activity. It has been found possible to obtain cytochrome oxidase

in 'solution' as a bile-salt complex (240) and it is to be hoped that this finding will be of aid in the further study of this important enzyme.

Calvin and his associates (31) and Hearon and colleagues (83, 84) have studied the properties of a group of synthetic metal chelates having the ability to oxygenate reversibly. The systems studied by Calvin have involved cobalt complexes with either an aldehyde or ketone and an amine. The sytem of Hearon (cobaltodihistidine) is of more immediate physiological interest since it involves histidine. Hearon *et al.* have presented a detailed analysis of the configuration and properties of cobaltodihistidine and its oxygenated form in terms of modern structural theory and have pointed out the relationship to the heme proteins. The special and unique ability of the imidazole group to exist in the form of different resonance hybrids and the contribution of the latter to stabilization of oxygenated complex are strikingly shown. The importance of these model systems for understanding of the mechanism of oxygenation is so obvious it need hardly be stressed. The papers of Hearon *et al.* may be commended as models for investigations on biological chelate complexes, since a variety of techniques and approaches were brought to bear on the problem. Recently a protein of blood plasma, siderophilin, has been found to be capable of binding iron ions (57, 186). Under certain conditions this iron-protein complex may bind carbon dioxide and oxygen.

Bücher and colleagues, in Warburg's laboratory, have described experiments on the properties of a heme protein which may possibly be of the greatest chemical and biological significance. They have measured the quantum efficiency of the photodissociation of carbon monoxide myoglobin using not only visible wave lengths (29) but also the ultra violet (28), especially at 280 mμ where the tyrosine and tryptophane of the protein show maximal absorption. The quantum yield was found to be 1.0, within experimental error, *at all wave lengths tested.* The conclusion drawn was that the action of absorbed light quanta in causing dissociation is independent of the site of absorption in the myoglobin molecule. The important implication is that some mechanism must exist to allow passage of energy absorbed by the tyrosine and tryptophane residues distributed throughout the protein molecule to the susceptible iron-carbonyl linkage. It has been suggested that this phenomenon may be of considerable biological significance since it implies that proteins, or perhaps only heme proteins, have the property of electron conductivity under special circumstances (54, 61a, 189, 223, 249, 250). However Franck and Livingston (58) have discussed the physics of such phenomena and have pointed out that suggestions of biological significance may be premature since the phenomenon observed by Bücher may exist only as a special result of photochemical excitation and intervening fluorescence phenomena and is not necessarily an indication of a general ability of the protein to 'conduct' electrons through structural channels in the molecule. However, the so-called heme-heme interactions of hemoglobin (252) must be regarded as a strong and independent indication of conduction through the protein in view of recent conclusions on the structure of hemoglobin (184) unless it is postulated that the heme-heme interaction is intermolecular rather than intramolecular. Although experimentation on this problem is at present largely limited to the photochemical approach, the potential biological implications of such a conductivity mechanism are so great, especially on such ques-

tions as the mechanism of electron transport via the cytochrome system (223) as well as the mechanism of heme-heme interactions in the polyheme proteins, that further efforts in this field will be awaited with great interest.

Although the foregoing discussion of the heme proteins is only superficial it is hoped that it will have served the purpose of pointing out some of the relationships between structure and catalytic function.

THE COPPER-ENZYMES

In addition to the well-recognized enzymatically active copper-proteins such as laccase, the phenol and polyphenol oxidases, and ascorbic acid oxidase, other copper-proteins with no known enzymatic activity have been obtained in highly purified form, such as hemocuprein and hepatocuprein (140) and the intensively studied hemocyanins (cf. 45), which are of great interest due to their functional resemblance to the hemoglobins and which may provide certain clues as to the mode of binding of the copper to the protein and especially the mode of interaction with oxygen. Reviews may be quoted for details on these enzymes (30, 45, 159). This discussion is limited to some general considerations on the role of the metal in catalysis.

The copper-oxidases catalyze the oxidation of their typical substrates by molecular oxygen without the accumulation of hydrogen peroxide (44, 216), although it has been suggested that hydrogen peroxide is formed and is used in secondary reactions (98). The activity of the copper oxidases is dependent on the copper content, which in highly purified preparations is of the order of 0.1 to 0.3 per cent. The oxidases are inhibited by sulfide, cyanide, and other metal-binding agents. Carbon monoxide inhibits the phenol oxidase of potatoes (120) and that of mushroom (108) but, significantly, laccase is not inhibited (109). These carbon monoxide inhibitions are not reversed by light and this is in agreement with the known properties of copper-carbonyls (168). In general the copper is tightly bound and survives dialysis against water; acids or treatment with cyanide, followed by dialysis, cause removal of copper from the protein. Reconstitution has been demonstrated by Kubowitz for the phenol oxidase from potatoes (120) although it was necessary to add back 10 times as much copper as was removed to restore full activity. Kubowitz found that a variety of other metal ions failed to reactivate the sytem although more recently it has been found that iron, cobalt and manganese will reactivate metal-free mushroom tyrosinase to a small extent depending on the reaction conditions (229). More recently Tissieres has demonstrated reconstitution of laccase (234). That copper is the active catalytic center of these oxidases is also indicated by the fact that model non-enzymatic catalyses of the autoxidation of ascorbic acid (15) (216, 247) and catechol (224, 245) by heavy metals are known. An essential difference between the models and the oxidases appears to lie in the fact that catalysis in the models in contradistinction to that by enzymes form hydrogen peroxide (79, 216, 247).

The copper catalysis probably involves a reversible Cu^{++}-Cu^{+} cycle in the case of both models and the oxidases, although this has been shown clearly for only phenol oxidase (120) among the copper enzymes. This enzyme is isolated in the cupric form. Kubowitz has shown that one mole of substrate (pyrocatechol) reduces one equivalent of Cu^{++}-enzyme to the Cu^{+} form forming one mole of o-quinone. The Cu^{+} form

of the enzyme was trapped as the carbon monoxide complex by carrying out the reaction under CO. Uptake of carbon monoxide was followed manometrically.

The relationship between color and valence state of the copper in the various copper proteins is not entirely clear. In the case of the phenol oxidase studied by Kubowitz (120) the enzyme is isolated in the cupric form and has only a faint yellow color. The spectrum showed no outstanding absorption other than the characteristic tyrosine-tryptophane type in the ultraviolet. Hemocyanins, which exist naturally in the cuprous condition, are blue when oxygenated (as in hemoglobin, the metal is probably in the reduced condition in the oxygenated complex) and colorless or only faintly colored when deoxygenated. It was at first presumed by Keilin and Mann (109) that the blue color of highly purified laccase was a property of the copper by analogy with the blue color of the oxygenated hemocyanins and hemocuprein (140) (hepatocuprein is colorless). However, more recently (111) they have found that highly purified laccase, which contains considerable carbohydrate material, could be split in such a way that the blue color of the enzyme was found in a copper- and protein-free fraction containing carbohydrate. The separated blue pigment could be reversibly reduced to the leuco compound. Keilin and Mann apparently did not attempt to reconstitute the active enzyme. In any event it appears possible that the blue color of laccase is not a function of copper content. Since hemocuprein (140) and ascorbic acid oxidase (174) are blue, it appears possible that in these cases also the color may not be a function of Cu content, or, if it is, it is conceivable that these proteins are isolated in an oxygenated condition. Although D. Bertrand has claimed to have obtained highly active laccase preparations free of the blue color and containing very little copper but significant amounts of manganese (21), these observations could not be confirmed by Tissieres (234) who has proved the activity of laccase to be dependent on copper by reconstitution experiments. The nature of the blue pigment found by Keilin and Mann is still unknown.

The findings of Keilin and Mann also bring up again the question of the nature of the linkage between copper and protein and the possible existence of organic copper-carrying prosthetic groups among the copper-proteins. The existence of an organic prosthetic group has long been a point of controversy as far as the hemocyanins are concerned, but now appears doubtful (cf. 45); Keilin and Mann could demonstrate no hematin in the polyphenol oxidase of mushrooms (108) or in laccase (109). Conant *et al.* (38) have brought forward some evidence that the copper of a hemocyanin is attached to a specific polypeptide fraction but the evidence has been criticized by Rawlinson (176) on what appear to be reasonable grounds.

Holmberg and Laurell have found that a copper protein of serum (92), similar to but not identical with hemocuprein, enhances the histaminolytic activity when added to serum in excess.

The existence of a very active copper ion catalysis of ascorbic acid autoxidation has raised some questions concerning the existence of a true ascorbic acid oxidase. Although Powers *et al.* (174) have isolated an ascorbic acid oxidase which shows about 1000 times the catalytic activity of its equivalent of cupric ion, it is known that complexes of copper with non-specific proteins enhance the activity of the copper under special conditions (45). More recently, Lampitt *et al.* (125, 126) have reopened this controversy with their description of new ascorbic acid oxidase models.

Weissberger *et al.* have studied the mechanism of copper ion catalysis of the autoxidation of ascorbic acid (246, 247). They have furnished evidence that the monovalent anion of ascorbic acid is the actual substrate of the catalysis and have have pictured the role of Cu^{++} as an electron acceptor as follows. The Cu^{++} ion is postulated as forming a coordination complex with the monovalent ascorbate ion and by an intramolecular electron shift the Cu^{++} is reduced to Cu^{+} and the ascorbate anion becomes a semiquinone radical. Since Cu^{+} does not readily coordinate with hydroxyl compounds it is released and oxidized by oxygen. The semiquinone radical may then take the reaction courses outlined by LuValle and Goddard (133), who have postulated general mechanisms for reduction of O_2 to either H_2O_2 or H_2O. Similar views were presented for the mechanism of metal-catalyzed catechol oxidation by the Szent-Györgyi school (13).

Study of the valence states of copper and the nature of the various complexes formed during enzymatic action or oxygenation by magnetic measurements, so useful for the iron porphyrins, is unfortunately limited in scope for the copper proteins due to the fact that the cuprous ion has no unpaired electrons and is thus diamagnetic; the cupric ion does have an unpaired electron and is paramagnetic. However, the unpaired electron of Cu^{++} is paired in most if not all of its coordination complexes.

Investigations on the mechanism of oxygenation of hemocyanins may throw considerable light on the mode of combination of oxygen with the copper oxidases during active oxidation; the oxygenated hemocyanin, although probably in the cuprous condition, may be a 'slow-motion' model of one phase of the true oxidase reaction sequence.

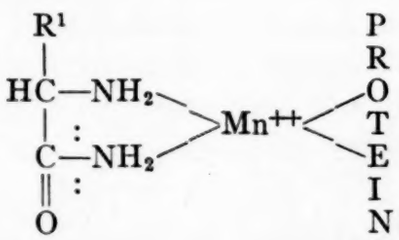
PROTEOLYTIC ENZYMES; ARGINASE

The first recorded observation of the activation of a proteolytic enzyme by metal ions appears to be that of Johnson *et al.* (101), who found that the rate of hydrolysis of certain leucyl peptides by dialyzed hog 'erepsin' was greatly increased in the presence of Mg^{++}. Since these early observations a number of activating effects of metal ions on peptidases have been studied (100, 208–210). Recently Smith (209, 210) has summarized the available facts on the activation of peptidases by metal ions and has postulated that the function of the metal ion is to form an asymmetrical chelate complex with the enzyme protein and the substrate. The polar groups of the substrate entering into the coordination were tentatively identified by means of substrate specificity studies. The complex is postulated as undergoing an 'electronic deformation' at the sensitive peptide linkage, rendering it susceptible to acid or base catalysis at physiological hydrogen ion concentrations. This hypothesis is very similar to that presented some years previously by Hellerman and Perkins for the role of metal in arginase action (88). With the exception of carboxypeptidase, none of the enzymes studied by Smith and his colleagues has been obtained in highly purified form and much of the work has been done on rather crude preparations and extracts. Nevertheless the hypotheses of Hellermann and Smith are of considerable interest since they represent an approach toward the understanding in chemical terms of metal activation of enzymes outside the pale of the classical, comparatively well-understood metalloenzymes such as the heme proteins. The peptidases showing metal activation are discussed largely in terms of the work of Smith and his colleagues; a discussion of arginase follows.

Leucine aminopeptidase, widely distributed in animal and plant tissues (17, 19), may be distinguished from other aminopeptidases in that it is very active against leucylglycine, leucylglycylglycine, and leucineamide but considerably less active against glycylglycine or alanylglycine. The activation by Mg^{++} first observed by Johnson *et al.* (101) was later studied more extensively in Johnson's laboratory (17) and it was found that Mn^{++} was much more effective than Mg^{++}. However, none of a large number of other cations (and anions) tested had any activating effect. Activating effects of Mn^{++} and Mg^{++} on the leucylpeptidase present in extracts of a wide variety of plant (18) and animal (19, 192, 201) tissues have been found. Smith has concluded that Mg^{++} and not Mn^{++} is the 'natural' activating ion in animal tissues (201).

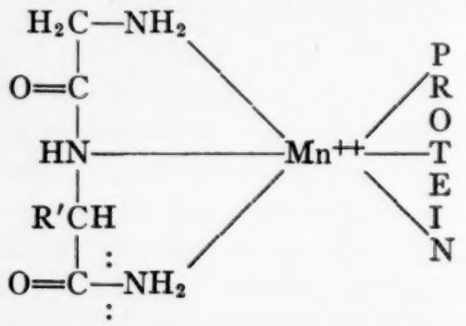
The work in Johnson's laboratory was confirmed and extended by Smith and Bergmann (198–200) and by Schwimmer (192). As the enzyme from hog duodenum (199, 200) was purified it became more dependent on the addition of Mn^{++} or Mg^{++} for activity. With material which appeared to be of about 40 per cent purity it was found that the activation by Mn^{++} (or Mg^{++}) was a very slow reaction, requiring several hours of contact of metal ion and protein (in the absence of substrate) for maximal activation. In fact the rate of activation was difficult to study since the metal-protein complex was far less stable than the metal-free protein. As a consequence, inactivation frequently accompanied activation by metal, complicating quantitative study of the kinetics. However, the data obtained by Smith (200) allowed calculation of the dissociation constant of the metal-enzyme (protein + Mn^{++} $\rightarrow$ (Mn^{++}-protein)), which was found to be of the order of 4×10^{-5}. Curiously, when Mn^{++}-activated leucylpeptidase was diluted with water and then tested for activity the data obtained indicated instantaneous dissociation of the Mn^{++}-protein complex, whereas the activation process is quite slow. Electrophoresis of an impure preparation showed that the mobility of a component suspected to be leucylpeptidase was slightly decreased in the presence of 0.01 M Mn^{++} (cf. 139).

Addition of the peptidase preparation to Mn^{++} caused striking changes in the Mn^{++} absorption spectrum and absorption changes were likewise observed when L-leucylglycine was added to Mn^{++} (204). These considerations have led Smith to postulate that the enzyme-substrate complex in this case (leucinamide as substrate) involves a coordination complex of Mn^{++}:



The sensitive bond is indicated by a dotted vertical line. Thus it is proposed that 2 chelate rings coordinated with Mn^{++} are formed. L-leucine aminopeptidase can also hydrolyze peptides such as glycyl-L-leucinamide at the leucine carbonyl group. Since the above formulation does not satisfactorily explain hydrolysis of such a peptide, Smith and Polglase (207) have suggested that in the case of the latter substrate a

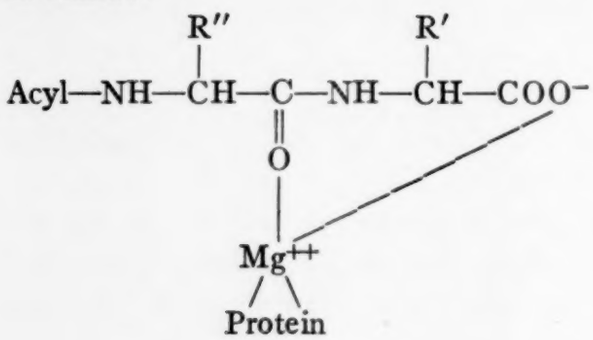
tridentate coordination complex of Mn^{++} is formed as follows:



After the formation of such metal-coordinated enzyme-substrate complexes Smith has suggested that the decreased activation energy of hydrolysis is produced by "the attraction of electrons by the metal ion," while the protein centers orient the substrate so that the net effect is an electronic deformation of the sensitive bond. The hydrolysis is pictured as being due, then, to the usual catalytic effects of hydrogen or hydroxyl ions. Coordination of metal with enzyme and substrate is viewed as reducing the activation energy sufficiently to cause 'acid-base' catalysis at pH values near neutrality.

Smith and Hanson have recently published evidence (206) that carboxypeptidase is actually a metal-protein, although a large amount of previous work on highly purified samples of this enzyme had not yielded an indication of this nature. They demonstrated that carboxypeptidase is inhibited by orthophosphate, cyanide, sulfide, citrate, oxalate and pyrophosphate. Inhibition by sulfide and cyanide was shown to be essentially irreversible by dialysis but treatment with horse methemoglobin, which is an extremely effective complexing agent for sulfide and cyanide (105), caused reactivation. However, inhibition by orthophosphate was instantaneously reversible on dilution. The inhibitory action of citrate, oxalate and pyrophosphate suggested that Mg^{++} was the active metal of carboxypeptidase. Smith and Hanson have tentatively identified Mg^{++} as the strongly bound metal ion present in carboxypeptidase since emission spectrography of the ash of highly purified enzyme indicated its presence (205, 206). All indications are that the magnesium is very strongly bound, and is not removed during the course of purification and crystallization. Kinetic studies revealed that one mole of phosphate or sulfide or 2 moles of cyanide combine with one active center in the inhibition process, and dissociation constants for each of these inhibitor-enzyme complexes were calculated.

Taking the extensive knowledge of the substrate specificity of carboxypeptidase into account, Smith and Hanson have proposed that the enzyme-substrate complex can be represented as follows:



In addition to the linkages shown it must be postulated that the substrate must also 'combine' with protein through the group R′, probably by van der Waals forces, since the character of R′ determines in a very high degree the specificity and rate of reaction. The group R″ is relatively unimportant. Smith has presented explanations for the observed inhibition of carboxypeptidase by D-amino acids, and also the relative inactivity of carboxypeptidase toward glycyl-L-tyrosine based on this hypothesis (210).

Johnson has isolated an apparently homogeneous protein from yeast which hydrolyzes tripeptides more rapidly than dipeptides (99). This enzyme is apparently not identical with the yeast aminopolypeptidase described by Grassmann (66, 67). The substrate split most rapidly by Johnson's enzyme is leucyldiglycine. This enzyme requires Zn^{++} for activity. The only other metal ion showing activation is Co^{++}, which is 60 per cent as effective as Zn^{++}. A unique property of this particular peptidase is that a halide anion is also required for activity. Nitrate activates to a small degree, but other anions are inactive. No attempts have been made to formulate the mode of action of Zn^{++} in the case of this enzyme nor has any quantitative work on apparent dissociation constants been reported.

It has been shown (142, 203) that glycyl-L-leucine is split by crude extracts of various animal tissues under circumstances suggesting that this activity (called glycyl-L-leucine peptidase) differs from that of other known peptidases. Zn^{++} appears to be the specific activating ion (142, 203), although in extracts of some tissues (203) Mn^{++} is more active than Zn^{++}, which may in these cases actually be inhibitory. Ca^{++} seems to be a general inhibitor and its effect is reversed by orthophosphate (203), suggesting a metallic ion antagonism although competition effects were not studied. It is curious that Smith (203) has found that some crude tissue extracts are instantaneously activated by the metal, whereas an ammonium sulfate fraction of intestinal mucosa possessing this activity showed a very slow rate of activation.

The splitting of glycylglycine by crude preparations of various cells appears to be catalyzed by a specific peptidase which requires Co^{++} as specific activating ion (17, 142, 143, 202, 204), Mn^{++} being somewhat less effective and other ions largely inactive. Zn^{++} is inhibitory (202). Contrary to the case of leucylpeptidase, the enzyme is stabilized somewhat in the presence of the metal. The dissociation constant of the hypothetical enzyme-metal complex has been approximated at 2.8×10^{-5}. Smith (204) has found that addition of glycylglycine and sarcosylglycine (which is also split by the enzyme) to Co^{++} caused large changes in the visible and ultraviolet spectrum of the latter, indicating formation of coordination complexes of Co^{++}. Certain glycine derivatives which were not split by the enzyme showed only very small changes in the Co^{++} spectrum, i.e. glycinamide, benzoylglycine, glycylsarcosine, etc. Glycylglycinamide, which is not split, did show a change in the spectrum, but not of the same type as that shown by glycylglycine. These findings have suggested to Smith a basis of substrate specificity and a possible mechanism of metal action, and he has proposed a chelate structure as the configuration of the enzyme-Co^{++}-substrate complex.

Carnosine (β-alanylhistidine) is split by extracts of certain tissues (80) and since the properties of carnosinase appear to differ from those of other peptidases it has been regarded as an enzymatic entity. The enzyme is inhibited by cyanide, sulfide,

cysteine, suggesting a metal as the active group. A partially purified preparation is activated by Mn^{++} or Zn^{++}, but Mg^{++}, Cd^{++}, Fe^{++}, Co^{++}, and Ca^{++} inhibit to some extent. The activation by Mn^{++} is a slow reaction, as in the case of leucine aminopeptidase. The enzyme is more stable in the presence of Mn^{++} than in its absence. There is a striking shift in the pH-activity curve of carnosinase with different metals, a phenomenon previously observed by Hellerman and Stock for arginase (89) which is discussed later.

The cleavage of glycyl-L-proline (59, 201) is catalyzed by extracts of several animal tissues; synthetic substrate studies suggest that a specific prolidase is involved. This enzyme is activated by Mn^{++} but not by other divalent ions. Here again, the rate of metal activation is quite slow.

The splitting of DL-alanylglycine and DL-leucylglycine by peptidases of *Leuconostoc mesenteroides* is accelerated by Zn^{++}, Pb^{++}, Cu^{++}, Mn^{++}, Sn^{++}, Cd^{++}, and Hg^{++} ions (16). The lack of metal specificity is noteworthy and suggests a possibly indirect mechanism of activation. The reservations of Mann and Lutwak-Mann (141) on interpretation of metal effects on enzyme preparations may be recalled here, as well as in other portions of this review. An effect of Mg^{++} has been noted under special conditions in the splitting of glutathione (22). Yudkin and Fruton (253) have observed a specific activation of a dialyzed preparation of dehydropeptidase of rat kidney by Zn^{++} (cf. 196).

Although it appears from the data reviewed above that most of the known exopeptidases are metal-activated, there are some exceptions. Smith and Bergmann (199) have found that a tripeptidase activity of intestinal mucosa showed no properties of a metal-enzyme and negative findings have also been observed with a similar enzyme from calf thymus (lymphopeptidase) (60), and a leucylpeptidase of rabbit skin (59).

No metal-activation effects on the well-known endopeptidases have been observed although in the light of the recent findings on carboxypeptidase mentioned above, a more thorough examination is warranted. Smith and Hanson (206) have mentioned unpublished work in which no inhibition of crystalline trypsin could be observed in the presence of a variety of metal-binding reagents. Smith has made the suggestion that even if no metal effects are found with the endopeptidases, the latter may be viewed as forming enzyme-substrate complexes much like those of the exopeptidases, with the exception that instead of a metal ion serving as coordination center, some highly polar organic grouping of the endopeptidase molecule may assume a similar function.

In evaluating Smith's hypothesis it must at once be recognized that the correlation between substrate specificity and the postulated sites of coordination in the substrate molecules is on the whole extremely impressive, as is the fact that the coordination sites are such as to yield, very conveniently, 5- or 6-membered rings. The experimental evidence for the hypothesis must still be regarded, however, as largely circumstantial and a number of criticisms might easily be made of the interpretation of some of the experimental findings and certain details of the hypothesis. Nevertheless the hypothesis on the whole is quite reasonable and it suggests a number of obvious experimental approaches for critical examination. Although the immediate

goal of Smith and his colleagues appears to be the survey of many different proteolytic enzymes for metal effects, it is to be hoped that they or others will be moved to concentrate efforts on a single highly purified peptidase showing metal activation and subject it and the hypothesis to a searching investigation.

Although arginase is not a peptidase, discussion of it in this section appears appropriate for reasons which become apparent. The literature on arginase and its activation by metals is extensive and is not quoted in full; the papers cited, however, provide a literature guide. Arginase has not yet been obtained in a highly purified state; some of the best preparations (11, 102, 151, 152, 177, 233) have been found to contain major protein impurities by electrophoretic methods. Such preparations are more or less reversibly activated by Mn^{++}, Co^{++}, Ni^{++} and Fe^{++}; Cd^{++}, Pb^{++}, Zn^{++}, Cu^{++}, Hg^{++} and Ag^{+} are either inactive or depress activity (48, 89, 151). Co^{++} is the most potent activating ion but the degree of activation finally attained (see below) is no greater than with Mn^{++} (151), which is regarded as the most probable physiological activator (25, 177, 197). Arginase present in crude tissue homogenates is not fully activated and the enzyme exists in widely different degrees of Mn^{++}-saturation in homogenates of different tissues (178). The activation has been found to be a very slow reaction (96, 151), the rate of which depends on time, temperature, metal ion and its concentration, and pH. With low concentrations of Mn^{++} at 20° full activation may require days. Mohamed and Greenberg (151) have suggested a process represented by the expression: Proarginase (inactive) + $Mn^{++} \rightleftharpoons$ arginase-Mn (active) although it is not clear whether they consider 'proarginase' as a biologically occurring zymogen in the same sense as pepsinogen or simply as the metal-free apoenzyme. In any event, it so happens that perhaps none of the enzymes now known to show a slow rate of metal activation has been obtained in physically homogeneous form, and it appears just barely conceivable that the slow rate of activation in some of these cases may be a reflection of a complex zymogen—active enzyme reaction involving two or more proteins. The slow rate of activation may, of course, be nothing more than the rate of a metal-coordinating reaction as Smith has suggested for leucine aminopeptidase.

The pH-activity curves of purified liver or jack bean arginase activated by different metal ions (Mn^{++}, Co^{++}, Ni^{++}) are markedly different, and it has been suggested by Hellerman and his colleagues that this phenomenon reflects in part the relative ability of a postulated metal-enzyme complex to form coordinate linkages with the substrate molecule (87–89, 219). Support for this view was furnished by pH-titration curves of arginine in the absence and presence of Co^{++}, Ni^{++} and Mn^{++}; Co^{++}, which causes a strong shift of the optimum pH of arginase to the acid side, forms a complex with arginine which has a much lower dissociation constant than that with Mn^{++}, which, when used as activating metal, shows a higher optimum pH. Therefore it appears as if the acid-base stability of the arginine-metal complex bears a relationship to the pH-activity behavior of the enzyme. The activating ions alone were found to have no catalytic effects on the hydrolysis of arginine, even at alkaline pH regions where hydroxyl ion catalysis is appreciable. Hellerman and his associates, in speculating on the significance of the above observations, have suggested that the role of the metal ion is to form a coordination complex uniting apoenzyme and sub-

strate. Furthermore, they have pointed out that the guanido group of arginine exists as the guanidinium ion at neutral or acid reactions. As the hydroxyl ion concentration is raised the resonance stabilization of the guanidinium ion is lost, due to loss of the proton, and the grouping is subject to OH^--catalyzed hydrolysis. Since the optimum pH of arginase action is in a pH region in which the guanidinium group is normally stabilized by resonance, they have suggested that the function of the metal and/or the apoenzyme is to destroy or modify the resonance stability of the guanidinium ion by forming a complex with it, thus exposing it in a form not stabilized by resonance to OH^- catalysis at a pH where it is normally stabilized by resonance. The approach to the problem made by Hellerman and his colleagues not only predates Smith's hypothesis on the role of metals in peptidases, but in some respects represents a deeper penetration.

Hunter and Downs have shown (97) that L-α-amino acids inhibit arginase but that D-α-amino acids are not inhibitory. It is of great interest that they found that monoamino acids are non-competitive inhibitors whereas the diamino acids, lysine and ornithine, are competitive inhibitors. Consideration of these data in the light of the approaches of Hellerman and Smith may become very revealing as to the nature and role of the coordination complexes involved in arginase action.

KETO ACID CARBOXYLASES, CARBONIC ANHYDRASE

Metal ions have been found to play a role in the action of certain enzymes concerned in decarboxylation of α- and β-keto acids. The biological important α-keto acid decarboxylations are those catalyzed by carboxylases, pyruvic acid oxidase and α-ketoglutaric oxidase. The carboxylase of yeast has been brought to a high state of purity by Green *et al.* (69) and Kubowitz and Lüttgens (121). The latter found that their purest preparations contained one mole of diphosphothiamine and one of Mg^{++} per mole of protein of assumed molecular weight of 75,000. Green *et al.* have found that the Mg^{++} is tightly bound; it can be dissociated irreversibly by exhaustive dialysis against water and reversibly by ammoniacal ammonium sulfate. Other divalent metals substitute for Mg^{++} qualitatively but only Mn^{++} is as effective as Mg^{++}. The rate of the carboxylase reaction is dependent on the order of addition of the cofactors to the dissociated apoprotein; the optimum order is enzyme, Mg^{++}, and diphosphothiamine. This suggests that Mg^{++} serves as the binding agent between protein and diphosphothiamine, although Smith has recently suggested the structure protein-diphosphothiamine-Mg^{++}, where the Mg^{++} would be in a more favorable position to be coordinated with the substrate in accordance with his views on the role of metals in peptidases (210). No more detailed information is available on the role of the metal ion in yeast carboxylase.

The α-keto acid carboxylases have also been observed in animal tissues. Green *et al.* (71) have described the properties of a partially purified preparation from heart muscle containing pyruvic and α-ketoglutaric carboxylases and they have found these enzymes to require Mg^{++} (or Mn^{++}) and diphosphothiamine for activity. Here again the metal-protein linkage is rather tight, being split only at acid pH values which were quite destructive to the apoprotein. It appears conceivable that these animal tissue carboxylases may actually be components of the pyruvic acid (14, 70, 130, 220) and α-ketoglutaric acid oxidase (163) systems of animal tissues which are

quite complex and still obscure and which may involve the interaction of phosphate. These complex α-keto acid oxidases also appear to involve Mg^{++} but in view of the scarcity of information, discussion of the role of the Mg^{++} appears fruitless at this time.

The enzymatic decarboxylation of β-keto acids offers a far more fertile ground for discussion. There are three β-keto acids of interest here, oxaloacetic acid, oxalosuccinic acid, and acetoacetic acid. In the case of oxaloacetic carboxylase, a number of preparations of the enzyme have been studied in detail, although probably none have been obtained in a homogeneous condition. Enzymes catalyzing the decarboxylation of oxaloacetate to pyruvate and CO_2 have been found in bacteria (104, 117, 173), animal tissues (53), erythrocytes (161) and in a number of plant tissues (213, 237, 239). Although oxaloacetic carboxylases from different sources differ somewhat in their activation by different metals, in each case Mn^{++} is the most active metal. The carboxylase from parsley root (213) is activated best by Mn^{++}. Co^{++}, Cd^{++}, Zn^{++}, Pb^{++}, Ni^{++}, Fe^{++}, Mg^{++}, Ba^{++} and Cu^{++} were also found to be active (in order of decreasing activity). The carboxylase from *Azotobacter vinelandii* (173) was found to be activated by Mn^{++}, Co^{++} and Zn^{++} and feebly by Mg^{++}. Ni^{++} and Fe^{++} were found inactive. These observations on the metal requirement have been made very significant from the standpoint of the reaction mechanism involved by the findings of Krebs (118) (cf. also 117, 162, 213) that a variety of polyvalent cations will catalyze non-enzymatic decarboxylation of oxaloacetate, thus providing a model catalytic system. Zn^{++}, Cu^{++}, Fe^{++}, Fe^{+++}, Al^{+++}, Pb^{++} (118), Co^{++} and Ni^{++} (213) were found very effective; Ca^{++}, Ba^{++}, Cd^{++}, Mg^{++} and Mn^{++} showed only feeble activity. Mn^{++}, although not a very active catalyst by itself, is the cation usually found most active in the carboxylases. None of these cations catalyzes a similar non-enzymatic decarboxylation of acetoacetate, mesoxalate, α-ketoglutarate or pyruvate. Acetonedicarboxylate is, however, split quite readily non-enzymatically in the presence of Al^{+++} to form acetoacetate and CO_2. This fact suggests that the β-keto acid must have two carboxyl groups for the metal ion catalysis. More recently Krebs and Eggleston have found (119) that acetoacetate is split non-enzymatically by Al^{+++} providing an α-keto acid is simultaneously present, a finding of some interest when reaction mechanisms are considered. They have suggested that acetoacetate and the α-keto acid may undergo a preliminary aldol condensation to form a dicarboxylic β-keto acid, a configuration which appears to be required for the catalytic action of Al^{+++}. Lynen and Scherer (134) and Ochoa (165) have found that the cations effective in decarboxylation of oxaloacetate are also effective in the non-enzymatic decarboxylation of oxalosuccinate. The optimum pH region of the catalytic action of these cations on oxaloacetic acid is about 4.0. The catalysis does not occur in strongly acid solutions.

Kornberg *et al.* (116) have investigated the role of the metal and the enzyme protein in β-keto acid decarboxylations employing spectrophotometric observation of what appeared to be complexes between Al^{+++} (or Mn^{++}) and β-keto acids (oxaloacetic, oxalosuccinic and acetoacetic acids). Although these acids (at pH 7.3) show negligible absorption above 250 mμ, in the presence of Al^{+++} complexes are formed showing high absorption peaks at 255 mμ (acetoacetate), 274 mμ (oxaloacetate)

and 252 mμ (oxalosuccinate). The spectrophotometrically observable complexes formed with oxaloacetate and oxalosuccinate were found to be unstable and disappeared as these compounds underwent catalytic decarboxylation; that formed with acetoacetate was stable (it will be recalled that Al^{+++} does not cause significant decarboxylation of acetoacetate). Mn^{++}, which is the cation required in the enzymatic decarboxylation, produced similar but relatively slight changes in the absorption spectra of oxaloacetate and oxalosuccinate. However these slight changes with Mn^{++} were greatly magnified in the presence of purified oxalosuccinic carboxylase, an effect which was in turn increased in the presence of high concentrations of neutral salts. The enzyme in the absence of Mn^{++} produced no such change. This intensified absorption was only transient and rapidly disappeared as the substrate was decarboxylated. Similar but far less marked changes accompanied addition of oxaloacetic carboxylase from *Micrococcus lysodeikticus* to oxaloacetate plus Mn^{++}. Kornberg *et al.* (116) interpreted their data to mean that the oxalosuccinic carboxylase catalyzes the formation of the Mn^{++}-oxalosuccinate complex, probably by forming an enzyme-Mn^{++}-oxalosuccinate complex which then is presumed to undergo decarboxylation. A similar picture was developed for the mechanism of oxaloacetic carboxylase action, although the absorption changes produced by addition of the enzyme were by no means as striking.

The interpretation placed on these spectrophotometric observations by Ochoa's group may not be correct in detail in view of recent work by Steinberger and Westheimer (214) who have studied the mechanism of the non-enzymatic decarboxylation of α,α'-dimethyloxaloacetic acid and its monoethyl ester. They have found that metal ions (Cu^{++}, Al^{+++}, Ni^{++}, Mn^{++}) catalyze the decarboxylation of the free acid but not the monoester (the carboxyl esterified was gamma to that ordinarily lost on decarboxylation). Since the α,α'-dimethyloxaloacetic acid cannot exist in the enol form, it is clear that the keto form undergoes decarboxylation. It would appear, then, that the metal ion catalysis of the decarboxylation requires the formation of a complex between the metal ion and the carboxyl group gamma to the one lost. Presumably the metal ion is also coordinated with the oxygen of the carbonyl group. Other observations (215) indicate that the ultraviolet-absorbing substances appearing when metal ion and substrate are mixed as in the experiments of Kornberg *et al.* (116) are not necessarily metal-substrate complexes but may be a mixture of complexes in which the complex of metal and the enolic form of the reaction product (i.e. α-ketoglutarate, pyruvate, etc.) may be predominant. The conclusion of Ochoa's group that the role of the enzyme is to catalyze formation of the metal-substrate complex is therefore not completely warranted by their data but not necessarily excluded. This case is one of the more promising instances in which research on model systems by physical-organic methods may ultimately be of the greatest importance in defining the role of the protein in enhancing the inherent catalytic properties of the active metal ions.

It should be pointed out that the oxaloacetic carboxylase of animal tissues, as it is now known, is probably a system of enzymes in which complex oxidation-reduction and phosphorylation phenomena also take place (145, 164, 236, 238).

No metal-activated acetoacetic carboxylase has been described, and since aceto-

acetate is not catalytically decarboxylated by metal ions, it may be predicted that a metal-activated carboxylase for this keto acid does not exist. Indeed, the only acetoacetic carboxylase known is the enzyme from *Cl. acetobutylicum* which Davies has brought to a high degree of purity (43). No metal is required for activity although a dialyzable cofactor is involved which the evidence indicated to be riboflavin phosphate. Primary amines are known to catalyze decarboxylation of β-keto acids (both mono- and dicarboxylic) (cf. 49, 172, 248) probably by a mechanism completely different from the metal ion catalysis although it appears not to be a general base catalysis (in terms of Bronsted's theory) (78). It will be of some interest if the isoalloxazine catalysis is also an amine-type catalysis.

Although carbonic anhydrase is not a keto acid carboxylase, the reaction catalyzed may be formally regarded as a reversible decarboxylation. Meldrum and Roughton found in their early study (146) that the enzyme is inhibited by cyanide and sulfide, indicating a dependence on a metal for activity. This indication was fully borne out by the work of Keilin and Mann (110) and others (95, 193) on highly purified carbonic anhydrase, which contains about 0.31 to 0.34 per cent Zn in tightly bound form. The metal dissociated readily only at very low pH values. Electrodialysis for 3 hours near neutrality was found not to remove Zn^{++} (193). Keilin and Mann have stated that the enzyme is the only zinc compound with carbonic anhydrase properties. They found inorganic zinc salts, simple organo-zinc compounds, zinc-porphyrin, crystalline zinc-insulin and Zn^{++} complexes of serum and tissue proteins to be devoid of this catalytic activity. These negative findings are of some interest in that certain other types of compounds are known to have catalytic activity in the hydration of CO_2 or the dehydration of carbonic acid, although none involve metals. Among these are borate, sulfite, selenite, arsenite (182), bromine and chlorine (but not iodine), hypobromite and hypochlorite (but not hypoiodite), hydrogen peroxide (113), and various organic bases such as histidine (113, 138, 182). These catalyses are not similar to that of carbonic anhydrase in mechanism nor do they approach it in magnitude. These substances probably exert their effects because they are proton acceptors (generalized base catalysis in Bronsted's terms) (183). In this connection, Roughton and Booth have shown that many anions are inhibitory to the enzymatic catalysis (182). Since the demonstration of the presence of the enzyme in gastric mucosa by Davenport (42), many authors have remarked on the correlation of carbonic anhydrase distribution with cellular functions concerned with acid-base balance and anion exchange (42a). The basic mechanism of the role of Zn^{++} in carbonic anhydrase has not been studied, although Smith has suggested that the Zn^{++} may function to unite enzyme and substrate in a coordination complex (209).

PHOSPHATASES; PHOSPHATE-TRANSFERRING ENZYMES

Virtually all the well-studied phosphatases and phosphate-transferring enzymes have been found to show either an absolute requirement for a metal or at least activation by a number of metal ions. Mg^{++} is usually the active metal (except in the actomyosin system, which is activated by Ca^{++} and inhibited by Mg^{++}), and positive effects have also been found with other divalent metals. In this class of enzymes a number of striking antagonisms between metallic ions have been observed; the an-

tagonism between Ca^{++} and Mg^{++} in the case of actomyosin appears to be involved in the physiological control of this system.

As a class, the phosphatases (actomyosin is discussed later) are not well understood from the standpoint of metal activation since only a very few of the many types of phosphatases have been obtained in a high state of purity. Tauber (227) has presented the most recent review of the phosphatases and has provided a classification of their properties (see also 155, 180). Erdtmann (52) was apparently the first investigator to demonstrate metal activation of a phosphatase in 1928.

The acid phosphatase of the prostate has been shown by Ohlmeyer (166) to be activated by Mg^{++} following exhaustive dialysis or electrodialysis. The enzyme was found to be inhibited by NaF and this inhibition was enhanced by Mg^{++}, Ca^{++}, Zn^{++}, Cd^{++}, Mn^{++}, Fe^{++}, Co^{++}, Ni^{++} and Cu^{++} under conditions in which the metals alone did not activate the enzyme. Ohlmeyer suggested that an inactive enzyme-metal-NaF complex is formed. An acid phosphatase from potatoes (86) has been found to be inhibited by NaF, but no activating effects by Mn^{++} or Mg^{++} were found.

Bailey and Webb (12) have isolated a phosphatase from yeast specific for pyrophosphate. This appears to be the only phosphatase yet obtained in electrophoretically homogeneous condition. It is activated by Mg^{++} but not by Ca^{++}, Mn^{++}, Zn^{++} or Be^{++}. The dissociation constant for the Mg-protein has been approximated at 0.001 M. Ca^{++} inhibits the enzyme competitively with respect to Mg^{++}; at a Ca/Mg ratio of 1/10, the inhibition is 65 per cent.

The alkaline phosphatases present in kidney and intestinal mucosa have been intensively studied and despite considerable effort (1, 2, 7, 10, 188, 232) have not been obtained in homogeneous condition. It has been apparent for some time that they are activated by Mg^{++} but the question has been obscured by indications (36) that a second metal (Zn^{++} or Co^{++}) may be required and by evidence that a dialyzable organic cofactor is required in addition to Mg^{++} (3, 6, 8). Activating effects have been observed with Co^{++}, Mn^{++}, Ni^{++} and Ca^{++}, in addition to Mg^{++} (160); inhibitory effects have been observed with Sr^{++}, Ba^{++}, Al^{+++}, Ag^{+}, Pb^{++}, Hg^{++} (160) and with Be^{++} (47, 77, 114). The inhibition by Be^{++} occurs at very low concentrations (10^{-6} M) and is not reversed by Mg^{++} but curiously, may be reversed by Mn^{++}, Co^{++} and Ni^{++} (47). Be^{++} also inhibits the Ca^{++}-activated ATP-ase (47). Competitive inhibition of Mg^{++}- or Ca^{++}-activated enzymes or physiological replacement of these metals by Be^{++} may be the basis for the rachitogenic and sarcomagenic activities of beryllium salts.

A specific hexosediphosphatase has been found to require Mg^{++} (64).

One of the most significant developments in modern biochemistry has been the demonstration of the relationship of the mechanical changes in the contractile elements of skeletal muscle to enzymatic events afforded by the work of the schools of Engelhardt (51) and Szent-Györgyi (223). Although the details of these experimental developments cannot be discussed here, this work has revealed that both the physical state (viscosity, birefrigence, i.e. the shape of the actomyosin molecule) and enzymatic (ATP-ase) activity of the actomyosin complex are highly dependent on the presence and relative concentrations of the alkali and alkaline earth ions. The general significance of this work is not restricted to the contractile elements of skeletal muscle;

Needham and his colleagues have pointed out the importance of contractile phenomena within certain types of cells as they relate to morphogenesis (41, 128, 129).

Although the mechanism of interplay of the various ions in the action of the actomyosin system during contraction and relaxation as proposed by Szent-Györgyi (223) has been criticized (26, 153, 154) the evidence still points to the fact that Ca^{++} and Mg^{++} ions are antagonistic in their effects on the activation of the ATP-ase action of the actomyosin complex. Mommaerts (153, 154) and Braverman and Morgulis (26) have published detailed studies of this antagonism. The former investigator demonstrated competitive inhibition of the Ca^{++}-activated ATP-ase activity of actomyosin by Mg^{++}, reporting that a Mg^{++}/Ca^{++} ratio of 0.2 yielded an inhibition of over 90 per cent. The latter investigators have found, under somewhat different conditions, (they used Ca^{++} and K^{+} comparable to the known concentrations in rabbit muscle; Mg^{++} was varied) that the inhibitory action of Mg^{++} was somewhat less than that found by the former group. Although the significance of this antagonism in the *in vivo* action of the contractile mechanism is not yet clear in detail, it appears certain that it will be found to play a vital role. The possible relationship of these effects to the classical 'calcium-rigor' of the perfused heart seems obvious. The Ca^{++}-Mg^{++} antagonism in myosin or ATP-ase action has been invoked as the mechanism underlying Mg^{++} anesthesia and its reversal by Ca^{++} (46) but this view has been criticized (76).

Evidence has been presented that the known rates of ATP-splitting during muscular contraction (intact muscle) (153, 154) or in muscle homogenates (26) cannot be accounted for by the actomyosin present, even during maximal Ca^{++}-activation. The enzyme recently described by Kielley and Meyerhof (112) may possibly account for a large part of the ATP-ase activity of muscle. This enzyme, which was obtained in a highly purified although apparently readily sedimentable (18,000 x g) form, is activated by Mg^{++} (to a lesser extent, Mn^{++}), rather than Ca^{++}, which actually was found to be a competitive inhibitor. Skeletal muscle therefore contains two separate ATP-ase activities in which the Ca^{++}-Mg^{++} antagonism is opposite in effect. The relationship of the Mg-activated enzyme to the contractile process is not clear as yet.

In addition to the phosphatases, a number of phosphate-transferring enzymes concerned in glycolysis have been found to require metal ions for activity. Some of these have been brought to a high state of purity. Although the mechanism of action of the metal is not known it would appear that these enzymes might offer very well-defined systems for such investigations.

One of the most interesting of these enzymes is that catalyzing the transfer of phosphate from phosphoenolpyruvate to ADP. This enzyme was obtained in crystalline form from human muscle by Kubowitz and Ott (122) and was found to require Mg^{++} for activity. No further report was made on the metal specificity. Although Meyerhof *et al.* (149) had reported that this enzymatic reaction was not reversible in muscle extracts, Boyer *et al.* (24) later found that K^{+} (or NH_4^{+}) was required (as well as Mg^{++}) for the reaction phosphopyruvate + ADP—pyruvate + ATP in crude muscle extracts, a fact which suggested to Lardy and Ziegler (124) that the dialyzed muscle extract employed by Meyerhof *et al.* (149) may have been low or lacking in K^{+}, accounting for the apparent irreversibility. Lardy and Ziegler demon-

strated that the reverse reaction occurred in dialyzed muscle extracts in the presence of K^+ and this has since been confirmed with the partially purified enzyme by Meyerhof and Oesper (148) although no data on the K^+ requirement were presented. It would be extremely desirable to have extensive data on the metal requirement of this enzyme since this appears to be the only instance recorded in which a K^+ effect has been observed to occur in a highly purified homogeneous enzyme system (however see Muntz, 156) and the only instance in which 2 different metals are required for the activity of one enzyme catalyzing a single, well-defined reaction. The function of each metal, the interrelations between the two, and the potentialities of metal ion antagonism are questions of great interest. Since the K^+ concentration within the cells of mammalian tissues is maintained at a level higher than that of the K^+ in extracellular fluids, an active metabolic process would appear to be required for the selective distribution. This is in fact indicated by work on the relationship of K^+ to fermentation in yeast extracts. K^+ is known to accelerate the rate of fermentation (55, 156) and, furthermore, K^+ is known to pass into the yeast cell from the medium during the process of fermentation (39, 90, 181, 175). The role of this transphosphorylase in the energy-yielding glycolytic cycle appears suggestive in this connection.

Bücher has obtained the metal-requiring transphosphorylase catalyzing the reversible reaction between 1,3-diphosphoglyceric acid and ADP in crystalline form from yeast (27). Although Mn^{++} was found to be more active than Mg^{++}, Bücher has concluded that Mg^{++} must be the natural activator in the yeast cell since yeast contains very little Mn^{++} compared to the large amount of Mg^{++}. The dissociation constant of the Mg^{++}-enzyme complex was determined to be 2.5×10^{-4}, a figure which is of the same order of magnitude as the dissociation constants of the complexes of enzyme with ADP and 3-phosphoglycerate. The complexes of enzyme with diphosphoglycerate and ADP were less easily dissociated, the constant of the former being smallest, namely 1.8×10^{-6}.

Najjar has obtained phosphoglucomutase in crystalline form (158) and has found that although it has some residual activity in the absence of added metal it is activated some four-fold by Mg^{++}. Mn^{++} was also effective. NaF was found to inhibit the enzyme in the absence of inorganic phosphate; however, the data obtained indicated that inhibition was due to the formation of a $(Mg^{++})(F^-)^2$ (organic-phosphate) complex which competed with Mg^{++} for the enzyme. Najjar did not study the primary metal specificity further and did not notice the requirement of a second metal as observed by Stickland (218). Neither worker was then aware of the finding of Leloir *et al.* (131) that the presence of glucose-1,6-diphosphate was required for the reaction. With the identification of this cofactor it should be possible to examine the secondary metal effect in a definitive way.

Hexokinase of both yeast (20, 123) and animal tissues (37) requires the presence of Mg^{++}, as do phosphofructokinase (228), myokinase (103), and the arginine-ATP transphosphorylase (225). Mn^{++} also serves as activating ion in most of these enzymes.

Enolase, which is not strictly speaking a phosphatase or transphosphorylase, has an absolute requirement for either Mg^{++}, Mn^{++} or Zn^{++} (241). It is probable that Mg^{++} is the natural activating ion, especially in view of the fact that the metal-

enzyme complex has a relatively high dissociation constant (K_{diss} = about 10^{-3}). It is interesting that the enzyme was crystallized as the inactive Hg^{++} salt. The mechanism of inhibition of enolase by fluoride deserves special mention since it may prove to be a rather wide-spread phenomenon among phosphate-handling enzymes. The inhibition by fluoride requires the presence of inorganic phosphate. Variation of Mg^{++}, F^-, and $HPO_4^=$ concentrations revealed that the effect of fluoride could be best interpreted by the equation:

Mg-fluorophosphate + Mg-enolase = Mg-fluorophosphoenolase + Mg salt

At higher fluoride concentrations the reaction may be simpler:

Mg-fluoride + Mg-enolase = Mg-fluoroenolase + Mg salt.

A competitive inhibition of enolase by Ca^{++} and Sr^{++} has been observed (167) but no data were presented.

It may be of some significance that although the phosphatases and phosphate-transferring enzymes appear to show a general requirement for Mg^{++}, there are a number of enzymes acting upon phosphorylated compounds, but which do not remove or transfer phosphate, which do not apparently require metals. Enolase is the exception to the latter statement. Enzymes of the latter group include phosphorylase, aldolase (however cf. 242), triosephosphate isomerase and dehydrogenase, glycerophosphate dehydrogenase, hexose phosphate isomerase, 'zwischenferment' and sucrose phosphorylase. Although the meaning of this fact is not clear, it may possess some significance in the mechanism of enzyme-substrate interaction in these 2 groups of enzymes. It has appeared reasonable to assume that the characteristic phosphate group of the different substrates involved may be a polar-'anchoring' group. Possibly polar groups of the protein other than metallic groups (guanido, ϵ-amino groups) may provide a site of attachment of the phosphate group in the latter class of enzymes.

The reviewer could find no record of the catalysis of non-enzymatic hydrolysis of phosphorylated compounds by metallic ions. No systematic attempts appear to have been made to provide a chemical hypothesis for metal-activation in this class of enzymes in the same terms employed by Smith for the exopeptidases. It has already been mentioned that Mg^{++} and Ca^{++} are probably able to complex with organic phosphates and the experimental approaches used for study of such complexes have been indicated in a previous section. It should be pointed out, however, that the mere fact that complexing may take place between metal and phosphorylated compound does not necessarily provide a basis for substrate specificity unless other functional groups of the phosphorylated substrate are also involved in the formation of a complex ion. It may be far more difficult to provide a working hypothesis for the role of the metal in this class of enzymes due to rather rigid substrate specificities (among the phosphate-transferring enzymes) than was the case for the exopeptidases, for which the substrate specificities, although not always rigid, were at least reasonably well known and could be reasonably accounted for on the basis of the chelation hypothesis.

That enzymatic hydrolysis of esters other than phosphate esters may also be metal-activated is suggested by reports (144, 147, 157) that choline esterase has the properties of a metal-enzyme.

and 252 mμ (oxalosuccinate). The spectrophotometrically observable complexes formed with oxaloacetate and oxalosuccinate were found to be unstable and disappeared as these compounds underwent catalytic decarboxylation; that formed with acetoacetate was stable (it will be recalled that Al^{+++} does not cause significant decarboxylation of acetoacetate). Mn^{++}, which is the cation required in the enzymatic decarboxylation, produced similar but relatively slight changes in the absorption spectra of oxaloacetate and oxalosuccinate. However these slight changes with Mn^{++} were greatly magnified in the presence of purified oxalosuccinic carboxylase, an effect which was in turn increased in the presence of high concentrations of neutral salts. The enzyme in the absence of Mn^{++} produced no such change. This intensified absorption was only transient and rapidly disappeared as the substrate was decarboxylated. Similar but far less marked changes accompanied addition of oxaloacetic carboxylase from *Micrococcus lysodeikticus* to oxaloacetate plus Mn^{++}. Kornberg *et al.* (116) interpreted their data to mean that the oxalosuccinic carboxylase catalyzes the formation of the Mn^{++}-oxalosuccinate complex, probably by forming an enzyme-Mn^{++}-oxalosuccinate complex which then is presumed to undergo decarboxylation. A similar picture was developed for the mechanism of oxaloacetic carboxylase action, although the absorption changes produced by addition of the enzyme were by no means as striking.

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It should be pointed out that the oxaloacetic carboxylase of animal tissues, as it is now known, is probably a system of enzymes in which complex oxidation-reduction and phosphorylation phenomena also take place (145, 164, 236, 238).

No metal-activated acetoacetic carboxylase has been described, and since aceto-

acetate is not catalytically decarboxylated by metal ions, it may be predicted that a metal-activated carboxylase for this keto acid does not exist. Indeed, the only acetoacetic carboxylase known is the enzyme from *Cl. acetobutylicum* which Davies has brought to a high degree of purity (43). No metal is required for activity although a dialyzable cofactor is involved which the evidence indicated to be riboflavin phosphate. Primary amines are known to catalyze decarboxylation of β-keto acids (both mono- and dicarboxylic) (cf. 49, 172, 248) probably by a mechanism completely different from the metal ion catalysis although it appears not to be a general base catalysis (in terms of Bronsted's theory) (78). It will be of some interest if the isoalloxazine catalysis is also an amine-type catalysis.

Although carbonic anhydrase is not a keto acid carboxylase, the reaction catalyzed may be formally regarded as a reversible decarboxylation. Meldrum and Roughton found in their early study (146) that the enzyme is inhibited by cyanide and sulfide, indicating a dependence on a metal for activity. This indication was fully borne out by the work of Keilin and Mann (110) and others (95, 193) on highly purified carbonic anhydrase, which contains about 0.31 to 0.34 per cent Zn in tightly bound form. The metal dissociated readily only at very low pH values. Electrodialysis for 3 hours near neutrality was found not to remove Zn^{++} (193). Keilin and Mann have stated that the enzyme is the only zinc compound with carbonic anhydrase properties. They found inorganic zinc salts, simple organo-zinc compounds, zinc-porphyrin, crystalline zinc-insulin and Zn^{++} complexes of serum and tissue proteins to be devoid of this catalytic activity. These negative findings are of some interest in that certain other types of compounds are known to have catalytic activity in the hydration of CO_2 or the dehydration of carbonic acid, although none involve metals. Among these are borate, sulfite, selenite, arsenite (182), bromine and chlorine (but not iodine), hypobromite and hypochlorite (but not hypoiodite), hydrogen peroxide (113), and various organic bases such as histidine (113, 138, 182). These catalyses are not similar to that of carbonic anhydrase in mechanism nor do they approach it in magnitude. These substances probably exert their effects because they are proton acceptors (generalized base catalysis in Bronsted's terms) (183). In this connection, Roughton and Booth have shown that many anions are inhibitory to the enzymatic catalysis (182). Since the demonstration of the presence of the enzyme in gastric mucosa by Davenport (42), many authors have remarked on the correlation of carbonic anhydrase distribution with cellular functions concerned with acid-base balance and anion exchange (42a). The basic mechanism of the role of Zn^{++} in carbonic anhydrase has not been studied, although Smith has suggested that the Zn^{++} may function to unite enzyme and substrate in a coordination complex (209).

PHOSPHATASES; PHOSPHATE-TRANSFERRING ENZYMES

Virtually all the well-studied phosphatases and phosphate-transferring enzymes have been found to show either an absolute requirement for a metal or at least activation by a number of metal ions. Mg^{++} is usually the active metal (except in the actomyosin system, which is activated by Ca^{++} and inhibited by Mg^{++}), and positive effects have also been found with other divalent metals. In this class of enzymes a number of striking antagonisms between metallic ions have been observed; the an-

tagonism between Ca^{++} and Mg^{++} in the case of actomyosin appears to be involved in the physiological control of this system.

As a class, the phosphatases (actomyosin is discussed later) are not well understood from the standpoint of metal activation since only a very few of the many types of phosphatases have been obtained in a high state of purity. Tauber (227) has presented the most recent review of the phosphatases and has provided a classification of their properties (see also 155, 180). Erdtmann (52) was apparently the first investigator to demonstrate metal activation of a phosphatase in 1928.

The acid phosphatase of the prostate has been shown by Ohlmeyer (166) to be activated by Mg^{++} following exhaustive dialysis or electrodialysis. The enzyme was found to be inhibited by NaF and this inhibition was enhanced by Mg^{++}, Ca^{++}, Zn^{++}, Cd^{++}, Mn^{++}, Fe^{++}, Co^{++}, Ni^{++} and Cu^{++} under conditions in which the metals alone did not activate the enzyme. Ohlmeyer suggested that an inactive enzyme-metal-NaF complex is formed. An acid phosphatase from potatoes (86) has been found to be inhibited by NaF, but no activating effects by Mn^{++} or Mg^{++} were found.

Bailey and Webb (12) have isolated a phosphatase from yeast specific for pyrophosphate. This appears to be the only phosphatase yet obtained in electrophoretically homogeneous condition. It is activated by Mg^{++} but not by Ca^{++}, Mn^{++}, Zn^{++} or Be^{++}. The dissociation constant for the Mg-protein has been approximated at 0.001 M. Ca^{++} inhibits the enzyme competitively with respect to Mg^{++}; at a Ca/Mg ratio of 1/10, the inhibition is 65 per cent.

The alkaline phosphatases present in kidney and intestinal mucosa have been intensively studied and despite considerable effort (1, 2, 7, 10, 188, 232) have not been obtained in homogeneous condition. It has been apparent for some time that they are activated by Mg^{++} but the question has been obscured by indications (36) that a second metal (Zn^{++} or Co^{++}) may be required and by evidence that a dialyzable organic cofactor is required in addition to Mg^{++} (3, 6, 8). Activating effects have been observed with Co^{++}, Mn^{++}, Ni^{++} and Ca^{++}, in addition to Mg^{++} (160); inhibitory effects have been observed with Sr^{++}, Ba^{++}, Al^{+++}, Ag^{+}, Pb^{++}, Hg^{++} (160) and with Be^{++} (47, 77, 114). The inhibition by Be^{++} occurs at very low concentrations (10^{-6} M) and is not reversed by Mg^{++} but curiously, may be reversed by Mn^{++}, Co^{++} and Ni^{++} (47). Be^{++} also inhibits the Ca^{++}-activated ATP-ase (47). Competitive inhibition of Mg^{++}- or Ca^{++}-activated enzymes or physiological replacement of these metals by Be^{++} may be the basis for the rachitogenic and sarcomagenic activities of beryllium salts.

A specific hexosediphosphatase has been found to require Mg^{++} (64).

One of the most significant developments in modern biochemistry has been the demonstration of the relationship of the mechanical changes in the contractile elements of skeletal muscle to enzymatic events afforded by the work of the schools of Engelhardt (51) and Szent-Györgyi (223). Although the details of these experimental developments cannot be discussed here, this work has revealed that both the physical state (viscosity, birefrigence, i.e. the shape of the actomyosin molecule) and enzymatic (ATP-ase) activity of the actomyosin complex are highly dependent on the presence and relative concentrations of the alkali and alkaline earth ions. The general significance of this work is not restricted to the contractile elements of skeletal muscle;

Needham and his colleagues have pointed out the importance of contractile phenomena within certain types of cells as they relate to morphogenesis (41, 128, 129).

Although the mechanism of interplay of the various ions in the action of the actomyosin system during contraction and relaxation as proposed by Szent-Györgyi (223) has been criticized (26, 153, 154) the evidence still points to the fact that Ca^{++} and Mg^{++} ions are antagonistic in their effects on the activation of the ATP-ase action of the actomyosin complex. Mommaerts (153, 154) and Braverman and Morgulis (26) have published detailed studies of this antagonism. The former investigator demonstrated competitive inhibition of the Ca^{++}-activated ATP-ase activity of actomyosin by Mg^{++}, reporting that a Mg^{++}/Ca^{++} ratio of 0.2 yielded an inhibition of over 90 per cent. The latter investigators have found, under somewhat different conditions, (they used Ca^{++} and K^{+} comparable to the known concentrations in rabbit muscle; Mg^{++} was varied) that the inhibitory action of Mg^{++} was somewhat less than that found by the former group. Although the significance of this antagonism in the *in vivo* action of the contractile mechanism is not yet clear in detail, it appears certain that it will be found to play a vital role. The possible relationship of these effects to the classical 'calcium-rigor' of the perfused heart seems obvious. The Ca^{++}-Mg^{++} antagonism in myosin or ATP-ase action has been invoked as the mechanism underlying Mg^{++} anesthesia and its reversal by Ca^{++} (46) but this view has been criticized (76).

Evidence has been presented that the known rates of ATP-splitting during muscular contraction (intact muscle) (153, 154) or in muscle homogenates (26) cannot be accounted for by the actomyosin present, even during maximal Ca^{++}-activation. The enzyme recently described by Kielley and Meyerhof (112) may possibly account for a large part of the ATP-ase activity of muscle. This enzyme, which was obtained in a highly purified although apparently readily sedimentable (18,000 x g) form, is activated by Mg^{++} (to a lesser extent, Mn^{++}), rather than Ca^{++}, which actually was found to be a competitive inhibitor. Skeletal muscle therefore contains two separate ATP-ase activities in which the Ca^{++}-Mg^{++} antagonism is opposite in effect. The relationship of the Mg-activated enzyme to the contractile process is not clear as yet.

In addition to the phosphatases, a number of phosphate-transferring enzymes concerned in glycolysis have been found to require metal ions for activity. Some of these have been brought to a high state of purity. Although the mechanism of action of the metal is not known it would appear that these enzymes might offer very well-defined systems for such investigations.

One of the most interesting of these enzymes is that catalyzing the transfer of phosphate from phosphoenolpyruvate to ADP. This enzyme was obtained in crystalline form from human muscle by Kubowitz and Ott (122) and was found to require Mg^{++} for activity. No further report was made on the metal specificity. Although Meyerhof *et al.* (149) had reported that this enzymatic reaction was not reversible in muscle extracts, Boyer *et al.* (24) later found that K^{+} (or NH_4^{+}) was required (as well as Mg^{++}) for the reaction phosphopyruvate + ADP—pyruvate + ATP in crude muscle extracts, a fact which suggested to Lardy and Ziegler (124) that the dialyzed muscle extract employed by Meyerhof *et al.* (149) may have been low or lacking in K^{+}, accounting for the apparent irreversibility. Lardy and Ziegler demon-

strated that the reverse reaction occurred in dialyzed muscle extracts in the presence of K^+ and this has since been confirmed with the partially purified enzyme by Meyerhof and Oesper (148) although no data on the K^+ requirement were presented. It would be extremely desirable to have extensive data on the metal requirement of this enzyme since this appears to be the only instance recorded in which a K^+ effect has been observed to occur in a highly purified homogeneous enzyme system (however see Muntz, 156) and the only instance in which 2 different metals are required for the activity of one enzyme catalyzing a single, well-defined reaction. The function of each metal, the interrelations between the two, and the potentialities of metal ion antagonism are questions of great interest. Since the K^+ concentration within the cells of mammalian tissues is maintained at a level higher than that of the K^+ in extracellular fluids, an active metabolic process would appear to be required for the selective distribution. This is in fact indicated by work on the relationship of K^+ to fermentation in yeast extracts. K^+ is known to accelerate the rate of fermentation (55, 156) and, furthermore, K^+ is known to pass into the yeast cell from the medium during the process of fermentation (39, 90, 181, 175). The role of this transphosphorylase in the energy-yielding glycolytic cycle appears suggestive in this connection.

Bücher has obtained the metal-requiring transphosphorylase catalyzing the reversible reaction between 1,3-diphosphoglyceric acid and ADP in crystalline form from yeast (27). Although Mn^{++} was found to be more active than Mg^{++}, Bücher has concluded that Mg^{++} must be the natural activator in the yeast cell since yeast contains very little Mn^{++} compared to the large amount of Mg^{++}. The dissociation constant of the Mg^{++}-enzyme complex was determined to be 2.5×10^{-4}, a figure which is of the same order of magnitude as the dissociation constants of the complexes of enzyme with ADP and 3-phosphoglycerate. The complexes of enzyme with diphosphoglycerate and ADP were less easily dissociated, the constant of the former being smallest, namely 1.8×10^{-6}.

Najjar has obtained phosphoglucomutase in crystalline form (158) and has found that although it has some residual activity in the absence of added metal it is activated some four-fold by Mg^{++}. Mn^{++} was also effective. NaF was found to inhibit the enzyme in the absence of inorganic phosphate; however, the data obtained indicated that inhibition was due to the formation of a $(Mg^{++})(F^-)^2$ (organic-phosphate) complex which competed with Mg^{++} for the enzyme. Najjar did not study the primary metal specificity further and did not notice the requirement of a second metal as observed by Stickland (218). Neither worker was then aware of the finding of Leloir *et al.* (131) that the presence of glucose-1,6-diphosphate was required for the reaction. With the identification of this cofactor it should be possible to examine the secondary metal effect in a definitive way.

Hexokinase of both yeast (20, 123) and animal tissues (37) requires the presence of Mg^{++}, as do phosphofructokinase (228), myokinase (103), and the arginine-ATP transphosphorylase (225). Mn^{++} also serves as activating ion in most of these enzymes.

Enolase, which is not strictly speaking a phosphatase or transphosphorylase, has an absolute requirement for either Mg^{++}, Mn^{++} or Zn^{++} (241). It is probable that Mg^{++} is the natural activating ion, especially in view of the fact that the metal-

enzyme complex has a relatively high dissociation constant (K_{diss} = about 10^{-3}). It is interesting that the enzyme was crystallized as the inactive Hg^{++} salt. The mechanism of inhibition of enolase by fluoride deserves special mention since it may prove to be a rather wide-spread phenomenon among phosphate-handling enzymes. The inhibition by fluoride requires the presence of inorganic phosphate. Variation of Mg^{++}, F^-, and $HPO_4^=$ concentrations revealed that the effect of fluoride could be best interpreted by the equation:

Mg-fluorophosphate + Mg-enolase = Mg-fluorophosphoenolase + Mg salt

At higher fluoride concentrations the reaction may be simpler:

Mg-fluoride + Mg-enolase = Mg-fluoroenolase + Mg salt.

A competitive inhibition of enolase by Ca^{++} and Sr^{++} has been observed (167) but no data were presented.

It may be of some significance that although the phosphatases and phosphate-transferring enzymes appear to show a general requirement for Mg^{++}, there are a number of enzymes acting upon phosphorylated compounds, but which do not remove or transfer phosphate, which do not apparently require metals. Enolase is the exception to the latter statement, Enzymes of the latter group include phosphorylase, aldolase (however cf. 242), triosephosphate isomerase and dehydrogenase, glycerophosphate dehydrogenase, hexose phosphate isomerase, 'zwischenferment' and sucrose phosphorylase. Although the meaning of this fact is not clear, it may possess some significance in the mechanism of enzyme-substrate interaction in these 2 groups of enzymes. It has appeared reasonable to assume that the characteristic phosphate group of the different substrates involved may be a polar-'anchoring' group. Possibly polar groups of the protein other than metallic groups (guanido, ϵ-amino groups) may provide a site of attachment of the phosphate group in the latter class of enzymes.

The reviewer could find no record of the catalysis of non-enzymatic hydrolysis of phosphorylated compounds by metallic ions. No systematic attempts appear to have been made to provide a chemical hypothesis for metal-activation in this class of enzymes in the same terms employed by Smith for the exopeptidases. It has already been mentioned that Mg^{++} and Ca^{++} are probably able to complex with organic phosphates and the experimental approaches used for study of such complexes have been indicated in a previous section. It should be pointed out, however, that the mere fact that complexing may take place between metal and phosphorylated compound does not necessarily provide a basis for substrate specificity unless other functional groups of the phosphorylated substrate are also involved in the formation of a complex ion. It may be far more difficult to provide a working hypothesis for the role of the metal in this class of enzymes due to rather rigid substrate specificities (among the phosphate-transferring enzymes) than was the case for the exopeptidases, for which the substrate specificities, although not always rigid, were at least reasonably well known and could be reasonably accounted for on the basis of the chelation hypothesis.

That enzymatic hydrolysis of esters other than phosphate esters may also be metal-activated is suggested by reports (144, 147, 157) that choline esterase has the properties of a metal-enzyme.

METALS IN THE ACTIVITY OF OTHER ENZYMES

The 5 major classes of metalloenzymes discussed do not include all examples of metal activation known. However it does not appear warranted to include a complete compilation of all known metal effects since in some cases, although the requirement for a metal has been clearly demonstrated, the mechanism of the reaction is either complex or obscure, as in oxidative phosphorylation and in the enzymatic depolymerization of desoxyribonucleic acid, or in other cases, the evidence for metal-activation is based merely on observation of inhibition by substances known to form metal complexes. Although the importance of such observations is not to be minimized, it is clearly evident from this review that it is most desirable to have more detailed data.

Since this manuscript was prepared, the important papers of Bamann and Meisenheimer (*Ber.* 71: 1711, 1980, 2087 and 2233, (1938)) were called to the reviewer's attention. They have found that rare earth metal hydroxide gels in aqueous suspension catalyze the hydrolysis of phosphate esters, metaphosphate and pyrophosphate. Hydroxides of thorium, lead and manganese were also active. None of the Group II hydroxides were active. The catalytic phenomenon showed a pH optimum of 8 to 9. These observations imply that the special spatial arrangement of metal ions in the gels studied may be of great importance in their catalytic activity.

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